1647

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mark D. Scott, et al.	Examiner:	R. Hayes
Serial No.	09/323,765	Group Art Unit:	1647
Filed:	June 1, 1999	Docket No.	259.006US1
Title:	ANTIGENIC MODULATION OF CELLS		

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
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The following documents are hereby submitted:

- ☒ Third Replacement Appeal Brief to the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office (34 pages) (three copies)
- ☒ Transmittal Sheet
- ☒ Four references cited
- ☒ Return postcard

The fee for the Appeal Brief was paid for when the original Appeal Brief, dated August 1, 2005, was submitted to the USPTO. Please consider this a PETITION FOR EXTENSION OF TIME for sufficient number of months to enter these papers if an additional extension of time is deemed necessary by the Office. Authorization is hereby given to charge Deposit Account Number 50-1391 if such additional extension is necessary.

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CERTIFICATE UNDER 37 C.F.R. 1.8: The undersigned hereby certifies that this Transmittal Letter and the paper, as described herein, are being deposited in the United States Postal Service, as first class mail, with sufficient postage, in an envelope addressed to: Mail Stop Appeal Brief – Patents, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450 on 23 August 2006.

Mark A. Litman
Name

Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE US PATENT OFFICE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Mark D. Scott et al.	Examiner: R. Hayes
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Sir:

Applicants present this **THIRD REPLACEMENT BRIEF ON APPEAL** in triplicate to the US PATENT OFFICE BOARD OF PATENT APPEALS AND INTERFERENCES and **request a personal appearance before the Board. The fee for payment of the personal appearance will be paid upon receipt of the Examiner's Answer.**

The U.S. Patent and Trademark Office is hereby authorized to debit any costs and fees associated with this Petition to Deposit Account No. 50-1391.

CERTIFICATE UNDER 37 C.F.R. 1.8: The undersigned hereby certifies that this Transmittal Letter and the paper, as described herein, are being deposited in the United States Postal Service, as first class mail, with sufficient postage, in an envelope addressed to: MAIL STOP: APPEAL BRIEF - PATENTS, P.O. BOX 1450, Commissioner for Patents, Alexandria, VA 22313-1450 23 AUGUST 2006.

Mark A. Litman
Name

Signature

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REAL PARTY IN INTEREST

The real party in interest in this Appeal is the licensee of the full right, title and interest in this Application, Canadian Blood Services, 1800 Alta Vista, Ottawa, Ontario CANADA K1G 4J5.

RELATED APPEALS AND INTERFERENCES

The Appellant(s), the legal representative prosecuting this application and Appeal, and the assignee are not aware of any Appeals or Interferences that will directly affect or have a bearing on the Board's of Patent Appeals and Interferences decision in this pending Appeal.

STATUS OF CLAIMS

Claims 1-26, 28 and 31 are pending. Claims 27, 29-30 and 32-52 have been cancelled.

The amendment to claim 28, after final rejection, has been entered.

STATUS OF AMENDMENTS

An Amendment was filed after the Final Rejection on 20 July 2005 amending only claim 28 to remove a rejection under 35 USC112, second paragraph. The Amendment has been entered.

SUMMARY OF CLAIMED SUBJECT MATTER

Acute tissue rejection causes damage to tissue when antibody binding and complement fixation underlie the destruction of donor tissue. (Page 1, lines 22-29). Attempts to reduce the impact or occurrence of tissue rejection has focused on selection of compatible tissue (e.g., blood typing), chemical intervention to reduce rejection, and direct chemical blocking on the tissue (Page 1, line 26 through page 3, line 24). The use of adducts of materials with biactivated tresylPEG (polyethylene glycol) on a targeted materials has been specifically shown in the prior art (Page 4, lines 22-26). Improved methods of reducing or avoiding rejection are always desirable.

The present invention claims non-immunogenic cells and a method by which cells may be converted to a non-immunogenic status by covalent attachment of a hydrophilic, biocompatible, non-immunogenic providing compound or polymer, with the cell displaying increased viability as compared to cells having even the same hydrophilic group attached by other chemical associations and reactions (Page 5, lines 2-27; page 6, lines 3-12; and pages 27-32). A hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently is attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer. (Claim 1)

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1) **Claims 2-7, 18-21, 23-25, 28 and 31 have been rejected under 35 U.S.C. 102(e) as anticipated by Desai et al. (U.S. Patent No. 5,578,442) in view of Lin at al. (*1976*).**

2) **Claims 1, 4, 8, 10-16, 24 and 26 have been rejected under 35 U.S.C. 102(b) as anticipated by Francis et al. (WO 95/06058).**

3) **Claims 1-26, 28 and 31 have been rejected under 35 U.S.C. 103(a) as obvious over the combination of Desai et al. in light of Lin et al. (*1976*) and in view of Francis et al. (WO 95/06058)**

ARGUMENT

Solely for the purposes of expediting this Appeal and complying with the requirements of 37 C.F.R. 1.192(c)(7), the following grouping of claims is presented. This grouping is not intended to constitute any admission on the record that claims within groups may or may not be independently asserted in subsequent litigation or that for any judicial determination other than this Appeal, the claims may or may not stand by themselves against any challenge to their validity or enforceability.

The claims will be respectively grouped under the various rejections

1) **Claims 2-7, 18-21, 23-25, 28 and 31 have been rejected under 35 U.S.C. 102(e) as anticipated by Desai et al. (U.S. Patent No. 5,578,442) in view of Lin et al. (*1976*).**

Claims 2-7 and 24 shall stand or fall with the patentability of claim 2.

Claims 18, 28 and 31 shall stand or fall with the patentability of claim 18, this claim specifically reciting a linking group not specifically recited in earlier claims.

Claims 19-23 and 25 shall stand or fall together with the patentability of claim 19, based upon the recitation of the attachment of the covalent bond directly to the antigenic determinants.

2) **Claims 1, 4, 8, 10-16, 24 and 26 have been rejected under 35 U.S.C. 102(b) as anticipated by Francis et al. (WO 95/06058).**

Claims 1, 8, 15, 24 and 26 shall stand or fall with the patentability of claim 1.

Claim 4 shall stand or fall by itself, this claim reciting the absence of toxic bye-products, a limitation not present in other claims.

Claim 10 and 16 shall stand or fall with the patentability of claim 10, reciting a specific blocking group.

Claim 11 shall stand or fall by itself, reciting a specific blocking group.

Claim 12 shall stand or fall by itself, reciting a specific blocking group.

Claim 13 shall stand or fall by itself, reciting a specific position of attachment with regard to the cell.

Claim 14 shall stand or fall by itself, reciting a specific linking group.

3) Claims 1-26, 28 and 31 have been rejected under 35 U.S.C. 103(a) as obvious over the combination of Desai et al. in light of Lin et al. (1976) in view of Francis et al.

Claims 1, 3, 8, 15, 17, 24, 25 and 26 shall stand or fall with the patentability of claim 1.

Claims 2 and 9 shall stand or fall with the patentability of claim 2, reciting a specific degree and test for stability.

Claim 4 shall stand or fall by itself, this claim reciting the absence of toxic bye-products, a limitation not present in other claims.

Claims 5, 6 and 7 shall stand or fall with the patentability of claim 5, this claim differing from claim 1 in reciting a nuclear cell.

Claim 10 and 16 shall stand or fall with the patentability of claim 10, reciting a specific blocking group.

Claim 11 shall stand or fall by itself, reciting a specific blocking group.

Claim 12 shall stand or fall by itself, reciting a specific blocking group.

Claim 13 and 19-23 shall stand or fall with the patentability of claim 13, reciting a specific position of attachment with regard to the cell.

Claim 14 shall stand or fall by itself, reciting a specific linking group.

Claim 18 shall stand or fall by itself, reciting a specific position of attachment to the cell surface.

ASRGUMENTS OF APPELLANTS

1) Claims 2-7, 18-21, 23-25, 28 and 31 have been rejected under 35 U.S.C. 102(e) as anticipated by Desai et al. (U.S. Patent No. 5,578,442) in light of Lin et al. (1976).

The Claims (represented by Claim 2, which is also highlighted for emphasis, below) specifically recite that the composition includes:

2. A non-aggregating, non-immunogenic nuclear cellular composition in which at least 25% by number of nuclear cells in said composition remain viable for 96 hours consisting of:

a) a mammalian nuclear cell having a cell surface and antigenic determinants on said surface; and

b) sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer **covalently attached to said surface** so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer. (Emphasis added).

These recitations are absolute and clear limitations on every claim remaining in the application. That specific limitation must be taught by Desai et al. for this rejection to be tenable. Desai et al do not teach covalent bonding of a non-immunogenic compound on a virus particle surface.

There is no specific disclosure that has been cited in the Office Action which asserts that the linker molecule is covalently bonded to the virus particle. The disclosure of Desai et al. clearly shows both the limitation of "covalent bonding" and bonding to a "virus particle" limitation to be absent from the invention contemplated by Desai et al.

Absence of Covalent Bonding

On column 5, line 38 through column 6, line 60, Desai et al. clearly describe a method and composition which "associates" a polycationic species with the negatively charged cell surface (e.g., column 5, lines 55-60). The language and disclosure in this section clearly denotes and describes an ionic association of the polycationic composition which renders the cells non-immunogenic, Desai et al. repeatedly use language and

description consistent with only ionic associations and inconsistent with covalent bonding. Even the reaction mechanisms for removal of the polycation binding to the cells and tissue is clearly incapable of relating to covalent bonding. Note specifically column 5, lines 55-67. The anions used to remove the polycationic materials from the cell surfaces must have high ionic strength. To remove a material from the cell surface that had been covalently bonded, specific types of chemical activity would have to be described.

The same section also refers to the necessary concentration of anionic species to reverse polycation binding to cells or tissue (column 5, lines 61-67). This language is specifically consistent with the existence of ionic binding and is inconsistent with covalent bonding. The fact that there is never any disclosure of specific reactive groups and reactions between the linker molecule and the cell surface is a further indication of the absence of any teaching or disclosure of covalent bonding to the cell surface by the linker molecule. In the absence of any indication of the necessary groups and reaction conditions for covalent bonding, and the consistent reference to ionic associations and ionic methods of release, it is absolutely clear that Desai et al. do not teach covalent bonding of the linker molecule to the cell surface.

Desai et al. cannot sustain a rejection under 35 U.S.C. 102(e) against the claims. The reference does not teach covalent bonding of the linker molecule to the virus particle surface or even to a cell surface. Although the Office Action repeats its assertion of the inherent formation of covalent bonds with the surfaces of cells or tissues, these assertion do not survive any serious scientific evaluation of the underlying technology.

The evidence to the contrary is that if the listed acids were capable of inherently forming covalent bonds with cells and tissues (in the absence of enzymes or catalysts for that specific reaction), life as we know it would cease on the Earth. If these and the other available alpha-amino acids could covalently bond to cells and tissues without enzymatic activity (which is not present in the *in vitro* environment of Desai), the cells and tissues within the body would bond together. This would mean that blood cells would bond to

vascular walls (e.g., cause clots and strokes), would crosslink tissue (e.g., the lungs), and cause other undesirable activities within the human body. These acids are present in foods, supplements and by-products of digestion and would covalently bond to the surface of the stomach, intestines, and other organs.

The Examiner has referred to portions of the Desai et al. specification where "free radical polymerization" is referred to, and these discussions have been asserted to reflect covalent bonding **to the cell**. The referred to section referred to free radical polymerization of components in the composition to each other, and there is no free radical polymerization to a cell, which would kill the cell. This would clearly fail to effect the successful recitation in claim 2 of the level of viability of the cell. This inability of strongly reactive materials to allow viability to continue in cells is supported by the showing on pages 27-32 of the specification, wherein the milder covalent bonding effected by the method of the invention enhanced cell survivability over the more intense bonding of Francis. Desai does not show covalent bonding **to the cell**, and if the language of the disclosure is misinterpreted to reflect free radical polymerization to the cell, that cell, like the cells of Francis (as shown on pages 27-32 of the specification) would not have a high degree of viability as recited in claim 2. Note the cited sections of the specification that the examiner refers to:

> "Optionally, **the polycationic species** employed in the practice of the present invention **can be further modified with one or more functional groups capable of undergoing free radical polymerization**. Suitable functional groups for this purpose include unsaturated species capable of free radical polymerization, such as, for example, acrylate groups, vinyl groups, methacrylate groups, and the like. When cells or tissues are treated with such modified polycationic species, the graft copolymer can be further subjected to free radical polymerization conditions, thereby stabilizing the association of graft copolymer with the cell surface. In addition, the further crosslinking of the graft copolymer forms a highly

stabilized, immunoprotective coating of water-soluble polymer about the treated cell or tissue.

The stabilization referred to in this paragraph is not free radical polymerization to the surface, but rather increasing the molecular weight of the species. The paragraph clearly states that the graft copolymer is crosslinked, not that there is any reaction to the cell surface by free radical polymerization. Any attempt to interpret the statement in the manner inherently asserted by the rejection is to extend the teachings of the reference to technology neither taught, implied or inherent. There simply is no showing of covalent bonding to the surface of the cell. The constant and repeated removability of the "polycationic species" clearly establishes that there is no covalent bonding. The rejection is clearly in error and must be withdrawn.

In summary, with regard to all claims (which recite covalent bonding), there appears to be clear evidence that the listed polyamino acids do not spontaneously, within the environment presented by Desai, form covalent bonds with cells and tissues.

NOTE: There is no Lin et al. reference (1976). The actual In et al. reference of record, cited and provided on June 3, 2004 (Not June 1, 2004 as indicated) is Lin and Riggs, "Photochemical Attachment of *lac* Repressor to Bromodeoxyuridine-Substituted *lac* Operator by Ultraviolet Radiation," *Proc. Nat. Acad. Sci.* Vol. **71**, No. 3, pp. 947-951, March 1974 (with a communication date of October 27, 1973). The reference to Lin et al. (1976) having been confusing and misleading.

Refutation of Materiality and Relevance and Impact of Lin et al. (1974)

It is first to be noted that Lin et al. has been cited in combination with Desai under 35 USC 102(e) so that the Lin et al. reference can do no more than explain the inherency of the teachings of DFesai and can add absolutely no material content to suggest modification of the actual teachings of Desai. To that end, Lin provides nothing of substance and does not overcome the reasons given above for the failure of the rejection.

The basis of the citation of Lin is to imply and assert that UV-crosslinking forms covalent bonds. The failure of this reference is that this teaching of Lin et al. (1974) is

specific to linking protein to DNA. However, Cesai does not crosslink protein to DNA and the invention is not crosslinking protein to DNA. Rather, polymer units are covalently reacted to the surface of cells to form non-immunogenic functions to the cell. The polymerizable moieties discussed by Desai, **as noted above,** are not polymerized to the cell, but are polymerized to form a polymer and then the polymer is attached through ionic functions (forming an ionic bond, not covalent bond) with other species and possibly the cell surface. **Lin et al. (1974) is therefore ineffective as teaching any inherency in Desai as it is a non-equivalent process and phenomenon, and even if combined under 35 USC 103(a), which it has not been, offers no teaching that enables forming a covalent bond as recited in the claims.** The rejection is completely in error, with or without Lin et al. (**1974**).

Claims 2-7 and 24 shall stand or fall with the patentability of claim 2. As noted above, all of the claims fail to even show covalent bonding. There has been no showing either of the survivability recited in claim 2. That rate cannot be assumed, especially in view of the comparative showing in the specification (e.g., pages 27-32) evidencing the uniqueness and unobviousness of those results.

Claims 18, 28 and 31 shall stand or fall with the patentability of claim 18, this claim specifically reciting a linking group not specifically recited in earlier claims. Desai et al. do not show a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Desai et al. teachings.

Claims 19-23 and 25 shall stand or fall together with the patentability of claim 19, based upon the recitation of the attachment of the covalent bond directly to the antigenic determinants. Even given an erroneous conclusion that Desai et al. teach covalent bonding to the surface of the cell (which it **DOES NOT**), there is no teaching that Desai et al. show bonding directly to the determinants. These claims are therefore additionally novel over the reference.

6. Claims 1, 4-5, 7-8, 10-16, 4 and 26 have been rejected under 35 U.S.C. 102(b) as anticipated by Francis et al. (WO 95/06058).

Claims 1-11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Desai et al. in view of Francis et al. (WO 95/06058). This rejection is also respectfully traversed. Although Francis does apparently incidentally show the covalent bonding of a moiety (including PEG, the erythrocytes of Example 7) to the surface of a red blood cell, the bonding is done for the purpose of differentiating cells (so that they may be separated by ionic or electrostatic or other physical process), and only mammalian cells, as opposed to virus particles are bonded with the differentiating compound.

This rejection therefore fails because the combination of references fails to provide any motivation for the covalent bonding of compounds to the surface of nuclear or anuclear cells **with the provision of an anti-immunogenic effect**. Even with the teaching of Francis that compounds can be covalently bonded to mammalian cells (including red blood cells in Example 7), the specification specifically replicated the process of Francis et al., compared those cells to cells produced according to the present invention, and clearly established that the process of Francis et al. (**which was not intended to provide an anti-immunogenic** effect) did not produce an anti-immunogenic effect. In this regard, please note Example IX, and especially the conclusion on Page 30, lines 15-29, especially where it is stated in lines 25-29 that:

> "**As shown, CmPEG readily modifies the RBC** [red blood cell] **surface and confers immunocamoflage. In contrast, the TmPEG method as taught by Francis fails to significantly modify RBC and does not yield any protection from immune recognition (Figure 1).** (*emphasis added*)

All of the claims, in various language, effectively recited "A non-aggregating, non-immunogenic ... cellular composition..." Francis et al. has been shown to provide a cell composition that is **NOT** non-immunogenic. Francis et al. has therefore been shown to fail to anticipate the present invention. As direct, detailed, and uncontraverted

evidence has been provided that shows that Francis et al. fails to anticipate this critical language of the claims, the rejection is clearly in error and must be withdrawn.

There is no motivation to perform a non-immunizing activity on nuclear or anuclear cells and clear evidence that the process of Francis et al. cannot provide that activity. Without such ability or motivation, there is no underlying basis for the assertion of obviousness.

The point of this argument is that Francis, even if covalent attachment is shown, destroys or greatly reduces the viability of the cells, contrary to the teachings of the present invention. This result is consistent with the purpose of Francis, which does not seek to create viable cells with immunogenic properties, but merely intends to provide a method of separating cells by bonding weighted polymers to them to make them more easily separable. This gross material addition to cells to make them more distinguishable is not material or functionally related to the purpose of creating **viable immunogenic cells**. Having no intent at cell survival, Francis uses techniques that reduce cell viability to a degree (shown by comparison in the specification examples on page 27-32) that are outside the limits of all claims. The term viable, alone, is sufficient to show lack of anticipation between the teachings of Francis and the claimed invention.

Claims 1, 8, 15, 24 and 26 shall stand or fall with the patentability of claim 1. The novelty of these claims has been firmly established above.

Claim 4 shall stand or fall by itself, this claim reciting the absence of toxic bye-products, a limitation not present in other claims. The novelty of this claim has been established above. Additionally, the examples and accompanying descriptions on pages 27-32 show that Francis produces waste bye-products that damage the cells. The reference therefore clearly fails to anticipate the invention as claimed.

Claims 5, and 7 shall stand or fall with the patentability of claim 5, this claim differing from claim 1 in reciting a nuclear cell. The arguments for establishing the novelty of these claims is otherwise identical to those presented with respect to claim 1 above.

Claim 10 and 16 shall stand or fall with the patentability of claim 10, reciting a specific blocking group. Francis does not show a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Francis teachings.

Claim 11 shall stand or fall by itself, reciting a specific blocking group. This specific blocking group has not been asserted to be shown by Francis and is therefore novel.

Claim 12 shall stand or fall by itself, reciting a specific blocking group. This specific blocking group has not been asserted to be shown by Francis and is therefore novel.

Claim 13 shall stand or fall by itself, reciting a specific position of attachment with regard to the cell. Francis has not been found nor asserted to show covalent bonding directly to the determinants on the cell surface. The rejection must additionally fail for that reason.

Claim 14 shall stand or fall by itself, reciting a specific linking group. Francis does not show a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Francis teachings.

7. Claims 1-16, 28 and 31 have been rejected under 35 U.S.C. 103(a) as obvious over the combination of Desai et al. in light of Lin et al. (1976) in view of Francis et al.

This rejection is clearly in error, at least for the following reason. Desai et al. has been clearly established as failing to show covalent bonding of PEG to cell surfaces. The Francis et al. reference, showing a specific format for providing covalent bonding of PEG to a cell surface for a purpose other than providing non-immunogenicity, **fails to provide non-immunogenicity by his sole described method.** That fact has been established by direct comparison of the Francis et al. method and a method according to the practice of the invention. Therefore, even if the methods of Desai et al. and Francis et al. were

combined, they would not be expected to provide the properties recited in the claims. There is no predictability, motivation or assurance that any combined product or process of Desai in view of Francis could produce a viable cell without consideration of the teachings of the present Application.

The rejection is therefore clearly in error. Not only was the covalent bonding shown by Francis et al. not intended to provide non-immunogenicity, the actual effect of the process failed to provide non-immunogenicity. The combination therefore fails to show that the invention as a whole, including the resulting properties, are obvious. The rejection therefore fails to meet minimum statutory requirements to establish a *prima facie* case of obviousness. The rejection is in error and must be withdrawn.

Additionally, the purpose for the covalent bonding of compounds to mammalian cell shown by Francis is for a fundamentally different purpose than that shown by Desai. Desai requires the preparation of reversible, non-adhesive cells, while Francis is teaching the preparation of adducts of a polymer and a targeted material, which are shown to be differentiable (e.g., in solvents so that they separate). Although Desai does teach that his reversible attachment (ionic attachment) of can reduce aggregation, Francis appears to indicate that aggregation still occurs with both his inventive composition and with control compositions (Examples 3 and 4). There is no nexus between the two references that would allow their combination, even if they are proposed to be combined. In addition, with this fundamental difference in the objective of the two references, they would not be combined to motivate one skilled in the art to modify the surface of a viral particle, a process not taught in either reference.

The basis of the citation of Lin is to imply and assert that UV-crosslinking forms covalent bonds. The failure of this reference is that this teaching of Lin et al. (1974) is specific to linking protein to DNA. However, Desai does not crosslink protein to DNA and the invention is not crosslinking protein to DNA. Rather, polymer units are covalently reacted to the surface of cells to form non-immunogenic functions to the cell. The polymerizable moieties discussed by Desai, **as noted above,** are not polymerized to

the cell, but are polymerized to form a polymer and then the polymer is attached through ionic functions (forming an ionic bond, not covalent bond) with other species and possibly the cell surface. **Lin et al. (1974) is therefore ineffective as teaching any obviousness from the teachings of Desai as it is a non-equivalent process and phenomenon, and even if combined under 35 USC 103(a), Lin et al. (1974) offers no teaching that enables forming a covalent bond between the non-immunogenic responsive polymer and the cell as recited in the claims.** The rejection is completely in error, with or without Lin et al. (**1974**).

Claims 1, 3, 8, 15, 17, 24, 25 and 26 shall stand or fall with the patentability of claim 1. The arguments directly above reflect the basic position on this set of claims. Those arguments are also applicable to all other claims in the Application, even where additional novel and unobvious features are shown.

Claims 2 and 9 shall stand or fall with the patentability of claim 2, reciting a specific degree and test for stability. Extensive comparisons were provided in the specification on pages 27-32 which have not been given their technical respect. That evidence is compelling on the fact that the recited covalent bonding and the specific degree of viability (which is recited in these claims) has not been shown to be taught, obvious, enabled or otherwise available from the teachings of these references. These properties are clearly not inherent as the reprise of the Francis process shows a significantly lower viability rate. There is no legal basis for the continued assertion of unobviousness except by ignoring the data and examples or by applying unwarranted pejorative attacks on the examples.

Claim 4 shall stand or fall by itself, this claim reciting the absence of toxic bye-products, a limitation not present in other claims. The novelty of this claim has been established above. Additionally, the examples and accompanying descriptions on pages 27-32 show that Francis produces waste bye-products that damage the cells. The reference therefore clearly fails to anticipate the invention as claimed.

Claims 5, 6 and 7 shall stand or fall with the patentability of claim 5, this claim differing from claim 1 in reciting a nuclear cell. Patentability arguments are otherwise the same as those provided above for Claim 1.

Claim 10 and 16 shall stand or fall with the patentability of claim 10, reciting a specific blocking group. Neither Francis nor Desai et al. show a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Francis teachings.

Claim 11 shall stand or fall by itself, reciting a specific blocking group. Neither Desai et al. nor Francis show this specific blocking group. It has not been asserted to be shown by Francis or Desai and is therefore novel and unobvious.

Claim 12 shall stand or fall by itself, reciting a specific blocking group. Neither Desai et al. nor Francis show this specific blocking group. It has not been asserted to be shown by Francis or Desai and is therefore novel and unobvious.

Claim 13 and 19-23 shall stand or fall with the patentability of claim 13, reciting a specific position of attachment with regard to the cell. Neither Desai et al. nor Francis has been asserted to specifically show attachment at the determinant sites. In the absence of such a teaching in either reference, the rejection must fail.

Claim 14 shall stand or fall by itself, reciting a specific linking group. Neither Desai et al. or Francis shows a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Francis teachings.

Claim 18 shall stand or fall by itself, reciting a specific position of attachment to the cell surface. Neither Desai et al. or Francis shows a linking unit derived from a cyanuric chloride reaction product. This rejection is therefore further not anticipated by the Francis teachings.

This rejection is in error and must be withdrawn.

CONCLUSION

All rejections of record have been shown in detail to be in error. The rejection should be reversed and all claims should be indicated as allowable.

Applicants believe the claims are in condition for allowance and request reconsideration of the application and allowance of the claims. The Examiner is invited to telephone the below-signed attorney at 952-832-9090 to discuss any questions that may remain with respect to the present application.

Respectfully submitted,
MARK D. SCOTT, et al.

By Their Representatives,
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Date 23 AUGUST 2006 By _____
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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Box: APPEAL BRIEF - PATENTS, P.O. BOX 1450; Commissioner for Patents, Alexandria, VA 22313-1450 on 23 AUGUST 2006.

Name: Mark A. Litman Signature

APPENDIX - THE CLAIMS ON APPEAL

1. (PREVIOUSLY PRESENTED) A non-aggregating, non-immunogenic anuclear cellular composition consisting of:

a) a mammalian anuclear cell having a cell surface and antigenic determinants on said surface;

b) a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

2. (PREVIOUSLY PRESENTED) A non-aggregating, non-immunogenic nuclear cellular composition in which at least 25% by number of nuclear cells in said composition remain viable for 96 hours consisting of:

a) a mammalian nuclear cell having a cell surface and antigenic determinants on said surface;

b) a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently

bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

3. (PREVIOUSLY PRESENTED) A non-aggregating, non-immunogenic nuclear cellular composition having insufficient amounts of toxic materials within said composition to be toxic to nuclear cells within said composition consisting essentially of:

a) a mammalian nuclear cell having a cell surface and antigenic determinants on said surface;

b) a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

4. (PREVIOUSLY PRESENTED) A non-aggregating, non-immunogenic anuclear or nuclear cellular composition consisting of:

a) a mammalian anuclear or nuclear cell having a cell surface and antigenic determinants on said surface;

b) a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on

said anuclear or nuclear cell surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer, said composition being free of any by-products from the covalent attachment of said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said anuclear or nuclear cell surface.

5. (PREVIOUSLY PRESENTED) A non-aggregating, non-immunogenic cellular composition having insufficient amounts of toxic materials within said composition to be toxic to cells within said composition consisting essentially of:

a. a mammalian nuclear cell having a cell surface and antigenic determinants on said surface;

b. a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

6. (PREVIOUSLY PRESENTED) A viable, non-aggregating, non-immunogenic cellular composition consisting essentially of:

a) a mammalian nuclear cell having a cell surface and antigenic determinants on said surface;

b) a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

7. (PREVIOUSLY PRESENTED) A non-immunogenic cellular composition consisting essentially of:

a. a mammalian nuclear cell having a cell surface and antigenic determinants on said surface;

a sufficient amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer covalently attached to said surface so that recognition of said antigenic determinants on said surface is blocked by said covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer.

8. (ORIGINAL) The cellular composition of claim 1 wherein said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is a polyalkylene glycol.

9. (ORIGINAL) The cellular composition of claim 1 wherein said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is a methoxypolyalkylene glycol.

10. (ORIGINAL) The cellular composition of claim 1 wherein said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is a dextran.

11. (ORIGINAL) The cellular composition of claim 1 wherein said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is Ficoll.

12. (ORIGINAL) The cellular composition of claim 1 wherein said hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is arabinogalactan.

13. (ORIGINAL) The cellular composition of claim 1 wherein said linking moieties are covalently bonded to said antigenic determinants on said cell surface.

14. (PREVIOUSLY PRESENTED) The cellular composition of claim 1 wherein said cell is an anuclear cell and the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is covalently bonded to the nuclear cell through a unit derived from reaction of a cyanuric chloride linking group on the

covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to the cell surface.

15. (PREVIOUSLY PRESENTED) The cellular composition of claim 1 wherein said anuclear cell is a red blood cell.

16. (ORIGINAL) The cellular composition of claim 10 wherein the antigenic determinants comprise a blood group antigenic determinants.

17. (PREVIOUSLY PRESENTED) The cellular composition of claim 1 wherein said anuclear cell is a platelet.

18. (PREVIOUSLY PRESENTED) The cellular composition of claim 2 wherein said cell is a lymphocyte and the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is covalently bonded to the nuclear cell through a unit derived from reaction of a cyanuric chloride linking group on the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to the cell surface.

19. (PREVIOUSLY PRESENTED) The cellular composition of claim 2 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity

providing compound or polymer to said surface, said linking moieties are covalently attached to said antigenic determinants on said cell surface and said nucleated cell is a vascular endothelial cell.

20. (PREVIOUSLY PRESENTED) The cellular composition of claim 2 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said surface, said linking moieties are covalently attached to said antigenic determinants on said cell surface and said nucleated cell is a hepatic cell.

21. (PREVIOUSLY PRESENTED) The cellular composition of claim 2 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said surface, said linking moieties are covalently attached to said antigenic determinants on said cell surface and said nucleated cell is a neuronal cell.

22. (PREVIOUSLY PRESENTED) The cellular composition of claim 2 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said surface, said linking moieties are covalently attached to said antigenic determinants on said cell surface and said nucleated cell is a pancreatic cell.

23. (PREVIOUSLY PRESENTED)The cellular composition of claim 2 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said surface, said linking moieties are covalently attached to said antigenic determinants on said cell surface and said nucleated cell is an epithelial cell.

24. (PREVIOUSLY PRESENTED) A method of producing a non-immunogenic mammalian cell, said method comprising:

covalently attaching an amount of hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to the cell surface, directly or by means of a linking moiety, so that said hydrophilic, biocompatible, nonimmunogenicity providing compound or polymer blocks recognition of antigenic determinants on the cell surface and yields a non-immunogenic cell.

25. (PREVIOUSLY PRESENTED)The method of claim 24 wherein linking moieties covalently attach the hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to said surface, said linking moiety is covalently bonded to said antigenic determinants on said cell surface.

26. (ORIGINAL) The method of claim 24 wherein said cell is a red blood cell.

27. (CANCELLED)

28, (CURRENTLY AMENDED) The ~~method~~ cellular composition of claim 21 wherein said cell is part of a tissue or organ and the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is covalently bonded to the nuclear cell through a unit derived from reaction of a cyanuric chloride linking group on the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to the cell surface.

29. (CANCELLED)

30. (CANCELLED)

31. (PREVIOUSLY PRESENTED) The cellular composition of claim 1 wherein said cell is a platelet and the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer is covalently bonded to the nuclear cell through a unit derived from reaction of a cyanuric chloride linking group on the covalently bonded hydrophilic, biocompatible, non-immunogenicity providing compound or polymer to the cell surface.

32.-52. (CANCELLED)

Evidence Appendix Page(s)

Solely because the Notice of Non-Compliant Brief specifically requested the following statement, the copies of the **REFERENCES** of:

1) Francis WO 98/32466;

2) Desai (US Patent No. 5,578,442);

3) Desai WO 93/18649; and

4) Lin and Riggs, "Photochemical Attachment of *lac* Repressor to Bromodeoxyuridine-Substituted *lac* Operator by Ultraviolet Radiation," *Proc. Nat. Acad. Sci.* Vol. **71**, No. 3, pp. 947-951, March 1974 (with a communication date of October 27, 1973).

are included herewith. Appellants would like to point out that this material is not evidence, but cited references. Extrinsic evidence does not include the references cited by the Examiner. Rather, commentary to the Rules state:

Evidence appendix. An appendix containing copies of any evidence submitted pursuant to § § 1.130, 1.131, or 1.132 of this title or of any other evidence entered by the examiner and relied upon by appellant in the appeal, along with a statement setting forth where in the record that evidence was entered in the record by the examiner. Reference to unentered evidence is not permitted in the brief. See § 41.33 for treatment of evidence submitted after appeal. This appendix may also include copies of the evidence relied upon by the examiner as to grounds of rejection to be reviewed on appeal.

The cited references, in spite of the requirements of the Examiner which have been complied with, do not constitute extrinsic evidence as intended by this section.

It is still the position of the Counsel of record that no extrinsic evidence is relied upon in this Appeal. There is no evidence of record with which this Appeal must be concerned other than the arguments and the references themselves.

Related proceedings appendix page(s)

There are no related proceedings in any official court, board, judicial or quasi-judicial venue in the United States regarding this Application or any related application to the knowledge of Appellants and their Counsel.

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(57) Abstract

In accordance with the present invention, there are provided methods to render cells non-adhesive and/or non-immunogenic with respect to macromolecules typically encountered in culture media or in physiological media.

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GRAFT COPOLYMERS OF POLYCATIONIC SPECIES AND WATER-SOLUBLE POLYMERS, AND USES THEREOF

The present invention relates to methods for rendering cells non-adhesive. In another aspect, the present invention relates to methods for rendering cells non-immunogenic. In yet another aspect, the present invention relates to methods for the stabilization of liposomes. In a further aspect, the present invention relates to methods for the *in vitro* generation of neural networks.

BACKGROUND OF THE INVENTION

Water-soluble polymers, such as polyethylene glycols (PEGs), have been investigated extensively in recent years for use as biocompatible, protein repulsive, noninflammatory, and nonimmunogenic modifiers for drugs, proteins, enzymes, and surfaces of implanted materials. These characteristics have variously been attributed to a combination of properties of such polymers, e.g., nonionic character, water solubility, backbone flexibility, and volume exclusion effect in solution or when immobilized on a surface.

While extensive efforts have been made to render foreign substances, such as drugs, proteins, and the like, non-immunogenic employing water-soluble polymers such as PEG, the use of such polymers to render an individual cell non-immunogenic has not been considered in the art. If such polymers could be attached directly to a cell surface, then it is possible, due to the exclusion of proteins and macromolecules from the vicinity of the cell surface, that the cell as a whole may be rendered non-immunogenic. The ability to accomplish such attachment would be invaluable for a variety of treatment protocols.

It is known that mammalian cell membranes have a variety of negatively charged species on their exterior. For example, negatively charged proteoglycans (PGA), glycosaminoglycans (GAG), such as chondroitin sulfate and heparin sulfate, and negatively charged lipids have all been identified on cell exteriors. Not surprisingly, polycation species such as polylysine and polyornithine interact with negatively charged cell surfaces to form a strong ionic linkage. Unfortunately, polycations (such as polylysine and polyornithine) are known to be cytotoxic, even at fairly low concentrations. Polylysine, for example, has been used as a cell fixative, and has been shown to cause cell aggregation (e.g., with human platelets).

While water-soluble polymers, having found use in a variety of biological applications, would be ideal for use in treating cells to render them non-immunogenic, their generally non-ionic nature renders them substantially unable to bind to cell membranes. Thus, for example, treatment of cells with unmodified PEG was unable to confer a non-adhesive or protein repellant character on such cells.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have developed methods to render cells non-adhesive and/or non-immunogenic with respect to macromolecules typically encountered in culture media or in physiological media.

The methods of the present invention can be used for a wide variety of purposes, e.g., for the treatment of cells used for implantation (thereby avoiding the need for immunosuppressive therapy), for the preservation of organs outside the body while awaiting transplant, for modifying surfaces of materials which are to be exposed to various

components of physiological media, for the stabilization of liposomes (and prevention of their uptake by the reticuloendothelial system), and the like.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a method to render cells non-adhesive, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

As employed herein, reference to rendering cells "non-adhesive" means, in an *in vitro* setting, that cells do not stick to wells (e.g., glass, plastic, and the like), or other surfaces with which they come in contact. Instead, non-adhesive cells, as contemplated herein, spread and grow, yet remain in suspension. In an *in vivo* setting, "non-adhesive" refers to cells which do not adhere to biologically-encountered macromolecules or proteinaceous matrix (e.g., collagen matrix). As used herein, "non-adhesive" also refers to cells which have been rendered non-immunogenic, i.e., cells which are substantially non-susceptible to an immune response mediated by biological macromolecules.

"Contacting" of cells or tissues with graft copolymer compositions contemplated for use in the practice of the present invention is typically carried out *in vitro* at room temperature for a time in the range of about 0.01 up to 1 hour or longer in suitable physiological buffer (i.e., pH in the range of about 7.2-7.4; osmolarity of about 290 mOsm/kg) containing a concentration of at least about 0.005% of graft copolymer, with respect to the concentration of the polycationic species used for the preparation of the cell surface (e.g., polylysine). It is presently preferred to treat cells with a solution of graft

copolymer containing a concentration of graft copolymer in the range of about 0.05 up to 1.0%, with concentrations of graft copolymer in the range of about 0.1 up to 0.5% being especially preferred. Those of skill in the art recognize that as the molecular weight of the polycationic species is increased, a lower concentration (determined on the same basis as set forth above) of the graft copolymer is required to produce the same beneficial effect.

As employed herein, an "effective amount" of graft copolymer compositions contemplated for use in the practice of the present invention is an amount sufficient to render said cells non-adhesive to biological macromolecules, while leaving the cells viable (as determined, for example, by suitable staining techniques). In the case of specialized cells, such as islets, it is desirable for the treated cells to retain their unique function as well as viability (i.e., the ability of islets to respond to exposure to glucose by secretion of insulin). Typically, an excess of graft copolymer is used with respect to the negative charges present on the surface of the cells to be treated. The quantity of graft copolymer required will vary depending on the cell type being treated and the concentration of cells to be treated. Typically, in the range of about 10^2-10^8 cells/ml will be treated. For example, up to about 10^8 bacterial cells/ml, up to about 100,000 fibroblasts/ml, or up to about 50,000 islets/ml will be treated.

Copolymer compositions contemplated for use in the practice of the present invention comprise a polycationic species having water-soluble polymer chains grafted thereon. Polycationic species contemplated for use in the practice of the present invention are polycationic species having sufficient charge density to allow binding of the above-described graft copolymer to cells, and include:

polymers containing primary amine groups, secondary amine groups, tertiary amine groups or pyridinyl nitrogen(s), such as polyethyleneimine, polyallylamine, polyetheramine, polyvinylpyridine, and the like,

polysaccharides having a positively charged functionality thereon (e.g., chitosan),

polyamino acids, such as:

poly-L-histidine, poly-im-benzyl-L-histidine, poly-D-lysine, poly-DL-lysine, poly-L-lysine, poly-ϵ-CBZ-D-lysine, poly-ϵ-CBZ-DL-lysine, poly-ϵ-CBZ-L-lysine,

poly-DL-ornithine, poly-L-ornithine, poly-δ-CBZ-DL-ornithine,

poly-L-arginine,

poly-DL-alanine-poly-L-lysine;

poly(-L-histidine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-phenylalanine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

random copolymers of:

L-arginine with tryptophan, tyrosine, or serine;
D-glutamic acid with D-lysine;
L-glutamic acid with lysine, ornithine, or mixtures thereof;

and the like, as well as mixtures of any two or more thereof.

Presently preferred polycations for use in the practice of the present invention include polylysine (i.e., poly-D-lysine (PDL), poly-DL-lysine, poly-L-lysine (PLL), poly-ε-CBZ-D-lysine, poly-ε-CBZ-DL-lysine, or poly-ε-CBZ-L-lysine), polyornithine (i.e., poly-DL-ornithine, poly-L-ornithine or poly-δ-CBZ-DL-ornithine), and the like.

Polycationic species having a wide range of molecular weights can be employed in the practice of the present invention. Polycations having a MW in the range of about 200 up to 1,000,000 are suitable, with molecular weights in the range of about 1000 up to 100,000 preferred. Presently most preferred polycationic species for use in the practice of the present invention will have molecular weights in the range of about 5,000 to 50,000.

Optionally, the polycationic species employed in the practice of the present invention can be further modified with one or more functional groups capable of undergoing free radical polymerization. Suitable functional groups for this purpose include unsaturated species capable of free radical polymerization, such as, for example, acrylate groups, vinyl groups, methacrylate groups, and the like. When cells or tissues are treated with such modified polycationic species, the graft copolymer can be further subjected to free radical polymerization conditions, thereby stabilizing the association of graft copolymer with the cell surface. In addition, the further crosslinking of the graft copolymer forms a highly stabilized, immunoprotective coating of water-soluble polymer about the treated cell or tissue.

Free radical polymerization of the above-described modified polycationic species can be carried out in a variety of ways, for example, initiated by irradiation with suitable wavelength electromagnetic radiation (e.g., visible or ultraviolet radiation) in the presence of a suitable photoinitiator, and optionally, cocatalyst and/or comonomer. Alternatively, free radical polymerization can be initiated by thermal decomposition of a suitable free radical catalyst, such as benzoyl peroxide, azobisisobutyronitrile, and the like.

Photoinitiators contemplated for use in the practice of the present invention include such ultraviolet (UV) initiators as 2,2-dimethyl phenoxyacetophenone, benzophenones and ionic derivatives (for water solubility), benzils and ionic derivatives thereof, thioxanthones and ionic derivatives thereof; and visible photoinitiators such as ethyl eosin, eosin, erythrosin, rose bengal, thionine, methylene blue, riboflavin, and the like.

Cocatalysts are typically used when the excited state of the photoinitiator is quenched too rapidly to efficiently promote polymerization. In such a situation, a cocatalyst (also referred to in the art as a "cosynergist", "activator", "initiating intermediate" or "quenching partner") will generally be employed. Cocatalysts contemplated for use in the practice of the present invention include triethanolamine, methyl diethanolamine, triethylamine, arginine, and the like.

Water-soluble polymeric species contemplated for use in the practice of the present invention are water-soluble polymers capable of rendering polycations non-immunogenic and include non-ionic, water-soluble polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly(hydroxyethyl methacrylate) (pHEMA), poly(acrylamide), poly(vinyl pyrrolidone) (PVP), poly(ethyl

oxazoline), polysaccharides (such as, for example, starch, glycogen, guar gum, locust bean gum, dextran, levan, inulin, cyclodextran, agarose, and the like); as well as ionic, water-soluble polymers such as polyacrylic acid (PAA) or polysaccharides (such as, for example, xanthan gum, carageenan, hyaluronic acid, heparin, chitosan, pectin, and the like); as well as copolymers of any two or more of said water-soluble polymeric species. Presently preferred water soluble polymers employed in the practice of the present invention are polyalkylene oxides, such as polyethylene glycol (PEG).

Water-soluble polymeric species having a wide range of molecular weights can be employed in the practice of the present invention. Polymeric species having a MW in the range of about 200 up to 1,000,000 are suitable, with molecular weights in the range of about 500 up to 100,000 preferred. Presently most preferred polymeric species for use in the practice of the present invention will have molecular weights in the range of about 1000 to 50,000.

Optionally, the water-soluble polymeric species employed in the practice of the present invention can be further modified with one or more functional groups capable of undergoing free radical polymerization. Suitable functional groups for this purpose include unsaturated species capable of free radical polymerization, such as, for example, acrylate groups, vinyl groups, methacrylate groups, and the like. When cells or tissues are treated with such modified water-soluble polymeric species, the graft copolymer can be further subjected to free radical polymerization conditions, thereby stabilizing the association of graft copolymer with the cell surface. In addition, the further crosslinking of the graft copolymer forms a highly stabilized, immunoprotective coating of water-soluble polymer about the treated cell or tissue.

Free radical polymerization of the above-described modified water-soluble polymeric species can be carried out in the same manner as described above with respect to free radical polymerization of modified polycationic species.

Graft copolymers contemplated for use in the practice of the present invention are those wherein the polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species, up to a maximum of one grafted chain per repeat unit of said polycationic species. For example, when the molecular weight of the polycationic species falls in the range of about 20,000, it will typically have grafted thereon at least about 5 chains of said water-soluble polymer chain per chain of polycationic species; with in the range of about 10-20 chains of said water-soluble polymer chain per chain of said polycationic species being the presently most preferred level of grafting. Those of skill in the art recognize that with polycationic species having higher molecular weights, higher levels of grafting will be desirable, and that the above values for grafting levels should be increased accordingly. Similarly, with respect to the water-soluble component of invention graft copolymers, the use of higher molecular weight species will allow one to achieve substantially the same result while grafting fewer (water-soluble) chains per chain of polycationic species.

Preparation of the graft copolymers of the present invention can be carried out employing chemical techniques known by those of skill in the art. For example, the free hydroxyl groups of the water-soluble component can be activated to render such groups susceptible to nucleophilic displacement. Thus, the free hydroxyl groups of the water-soluble component can be subjected to esterification, etherification, amidation,

urethane formation, and the like. Such reactions involve the generation of such intermediates as carbonates, sulfonates, xanthates, epoxides, aliphatic aldehydes, carboxymethyl azides, succinimidyl succinates, and the like. The activated water-soluble component can then be coupled to a suitable polycationic species, for example, by nucleophilic displacement.

Cell types contemplated for use in the practice of the present invention include islets, fibroblasts, thyroid cells, parathyroid cells, adrenal cells, neuronal cells, dopamine secreting cells, hepatocytes, nerve growth factor secreting cells, adrenaline/angiotensin secreting cells, norepinephrine/metencephalin secreting cells, human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV, and the like.

Also included within the scope of the present invention are cells having a modified cell surface which is substantially non-adhesive with respect to macromolecules encountered in physiological environments.

In accordance with another embodiment of the present invention, there is provided a process to remove copolymer compositions contemplated for use in the practice of the present invention from cells treated as described above, said process comprising contacting such cells with an effective amount of an anionic species.

Anionic species contemplated for use in this embodiment of the present invention include monomeric or polymeric anions. Any soluble anionic species capable of reversing the association of polycationic species with negatively charged cell surface can be employed for this purpose. Presently preferred anionic species are polyanionic species, such as, for example, heparin, heparin sulfate, chondroitin sulfate, soluble alginates (e.g.,

sodium alginate, potassium alginate, ammonium alginate, and the like), bovine serum albumin, hyaluronic acid, pectin, carageenan, oxidized cellulose, and the like.

"Contacting" of treated cells to remove invention copolymer therefrom is carried out at room temperature for a time in the range of about 0.01 up to 1 hour or longer in physiological buffer solution containing anionic species at a sufficiently high ionic strength to reverse the association of polycationic species with negatively charged cell surface.

An effective amount of anionic species to employ in accordance with this embodiment of the present invention depends on the specific anionic species employed. Generally, the concentration of anionic species employed will be sufficient to reverse polycation binding to cells or tissues, but not so high as to be toxic to the biological material being treated. Concentrations employed are typically in excess of the amount of anion actually needed to disrupt binding of polycation to cell surface. Thus, for example, presently preferred treating solutions contain about 2.5 Units/ml of heparin or 2 mg/ml of bovine serum albumin.

In accordance with yet another embodiment of the present invention, there is provided a method to render cells non-immunogenic, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of cells with graft copolymer compositions to render cells non-immunogenic is typically carried out as described above with respect to rendering cells non-adhesive.

The process of the present invention can be used for rendering non-immunogenic any cell, tissue, organ, or system of organs, and the like, that may be used for transplant or the like.

Also included within the scope of the present invention are cells having a modified cell surface which is substantially non-immunogenic with respect to mediators of immune response, e.g., biological macromolecules such as proteins, enzymes, and the like.

In accordance with another embodiment of the present invention, there is provided a process to remove copolymer compositions contemplated for use in the practice of the present invention from cells treated as described above, said process comprising contacting treated cells with an effective amount of an anionic species, as described above.

In accordance with still another embodiment of the present invention, there is provided a method to preserve cells and/or tissues which are subjected to long periods of storage before being used for therapeutic applications, said method comprising contacting said cells and/or tissues with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of cells and/or tissues with graft copolymer compositions to preserve same is typically carried out as described above with respect to rendering cells non-adhesive and/or non-immunogenic.

In accordance with a still further embodiment of the present invention, there is provided a method for associating water-soluble polymer with a cell surface, said method comprising:

grafting water-soluble polymer onto a polycationic resin to produce a copolymer of said water-soluble polymer and said polycation, and thereafter

contacting said cell surface with an effective amount of said copolymer.

If desired, the copolymer can be substantially removed from the cell surface employing the above-described removal process.

In accordance with a further embodiment of the present invention, there is provided a method for the stabilization of liposomes having negatively charged surfaces, said method comprising contacting said liposomes with an effective, stabilizing amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

"Contacting" of liposomes for the stabilization thereof is carried out at room temperature for a time in the range of about 0.01 up to 1 hour or longer in physiological buffer.

An effective amount of graft copolymer for use in this embodiment of the present invention is an amount sufficient to render such liposomes essentially non-detectable *in vivo*, thereby reducing uptake of the liposome by the reticuloendothelial system (and increasing liposome circulation times *in vivo*). Suitable quantities of graft copolymer will render the liposomes substantially non-adhesive to biological materials, while leaving the liposome intact, and without adversely affecting the function and/or activity of the contents thereof, if any. Typically, a concentration of graft copolymer sufficient to neutralize the negative charges on the surface of the liposome will be employed. Concentrations in the range of

at least about 0.05% of graft copolymer, with respect to the concentration of the polycationic species used to treat the surface of said liposome will be employed; with concentrations of graft copolymer in the range of about 0.1 up to 0.5% being presently preferred.

One can readily determine the stability of a liposome using a functional assay, such as the following. In an *in vitro* setting, the stability of liposome-encapsulated hemoglobin in an un-modified liposome could be compared to the stability of hemoglobin encapsulated in a liposome stabilized in accordance with the present invention (i.e., the result of treating an un-modified liposome with a sufficient quantity of graft copolymer described above to stabilize the liposome). The release of hemoglobin into the surrounding buffer media over time would then be assayed, with an extended time-frame for release of hemoglobin indicating enhanced liposome stability.

In accordance with yet another embodiment of the present invention, there is provided a method for producing neural networks on a substrate, said method comprising:

masking that portion of said substrate which defines the desired network,

rendering the unmasked portion of said substrate non-adhesive by the above-described method of the invention,

removing the mask, then

allowing cells to spread and grow on said substrate, wherein cells grow only on the portion of the substrate which has not been treated with graft copolymer.

Substrates contemplated for use in the above-described method include tissue culture substrates, such as collagen, tissue culture polystyrene, microporous dextran substrate, and the like.

Masking contemplated by the above-described method can be accomplished in a variety of ways, such as, for example, by covering a portion of the substrate with an agent which does not serve as a substrate for cell growth (e.g., a piece of tape, or the like).

The masking agent employed can readily be removed by merely reversing the process employed for applying the mask to the substrate.

Conditions required for cells to spread and grow on the substrate are standard cell culture conditions.

The resulting neural networks can be used for a variety of purposes, such as, for example, for studying the transmission of nerve impulses, for connection between a nerve cell and an electrical circuit, and the like.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

EXAMPLE I

Synthesis of a Graft copolymer of Poly-L-Lysine and Polyethylene Glycol

Twenty grams (20 g) of PEG (molecular weight 10,000 g/mol, having the structure HO-PEG-OH) were dried in a vacuum oven at 80°C for 24 hours and dissolved in 100 ml of methylene chloride that had been dried by molecular sieves (4A). Then, 3.24 g of 1,1-carbonyldiimidazole (CDI, 5 fold excess, to ensure the activation of 100% of PEG end groups) were added to the solution and stirred overnight at room temperature in an argon atmosphere. The CDI-activated PEG was then precipitated with an excess of anhydrous diethyl ether and dried overnight under vacuum. Five grams

(5 g) of the CDI-activated PEG were dissolved in 20 ml of 5 mM sodium borate buffer (pH 9). In order to prevent crosslinking of poly-L-lysine (PLL) with the 100% CDI-activated PEG, 50% of the PEG end groups were inactivated by adding 30.2 μl of ethanolamine to the buffer solution and stirring for 4-6 hours at room temperature. This results in a mono-activated CDI-derivative of PEG, having the structure CDI-PEG-OH. Alternatively, a monomethoxy PEG could be used to avoid this partial deactivation step, but monomethyl PEGs are presently available commercially only up to molecular weight 5000.

Following the above-described partial deactivation step, 50 mg of PLL (M.W. 20,100 g/mol) were added to the reaction mixture and stirred for 24 hours at room temperature. The solution was then dialyzed for 24 hours against deionized water and freeze dried to obtain a powder. This procedure produced a PEG graft copolymer (PLL-PEG) having a concentration of approximately 10-20 PEG chains per PLL chain.

EXAMPLE II

Demonstration of Cell Binding Properties of PLL-PEG to Fibroblasts: Effect on Cells in Suspension

The cell binding effects of PLL-PEG copolymer produced as described in Example I were tested on cultures of human foreskin fibroblasts (HFF). These cells are anchorage dependent and ordinarily die within 4 to 10 hours if they do not adhere and spread on a surface. Thus, a flask of confluent HFF was harvested with trypsin-EDTA, then the resultant cells in suspension were split into 6 batches, each containing approximately 170,000 cells. Each batch was centrifuged to obtain cell pellets. Six different solutions were used for cell treatment:

(A) Fibroblast culture media: Dulbecco's modified Eagles' medium (D-MEM) containing 10% fetal bovine serum;

(B) 10 mM HEPES buffered saline (HBS), pH 7.4;

(C) HBS containing 0.1% (w/v) PLL;

(D) HBS containing 0.5% PEG (M.W. 10,000);

(E) HBS containing 0.1% PLL and 0.5% PEG; and

(F) HBS containing 0.3% PLL-PEG (based on PLL concentration).

All solutions were sterilized by filtration through 0.22 micron filters prior to use. The cell pellets were resuspended in 2 ml of solutions A, B, C, D, E or F for approximately 10 minutes. The tubes were then centrifuged (200 xg for 5 minutes), the solutions aspirated and replaced with fibroblast culture medium, and the cells plated onto culture dishes. The plated cells were observed periodically to verify adherence and spreading. The cells were also stained with trypan blue (TB) to test viability. Table I summarizes the observations over 5 days following the seeding.

TABLE I

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	A (fibroblast culture medium)	B (buffered saline)	C (PLL)	D (PEG)	E (PLL + PEG)	F (PLL-PEG copolymer)
1 hr	Normal spreading	Normal spreading	No adherence; Cell clumping	Normal spreading	No adherence; Cell clumping	No adherence; No clumping
% viability	95	95	0	90	0	80
24 hr	Normal spreading	Normal spreading	No adherence; Cell clumping	Normal spreading	No adherence; Cell clumping	No adherence; No clumping
% viability	100	100	0	95	0	70

TABLE I (cont)

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	A (fibroblast culture medium)	B (buffered saline)	C (PLL)	D (PEG)	E (PLL + PEG)	F (PLL-PEG copolymer)
48 hr	Confluent monolayer	Confluent monolayer	No adherence; Cell clumping	Confluent monolayer	No adherence; Cell clumping	No adherence; No clumping
% viability	100	100	0	95	0	60
120 hr	Confluent monolayer	Confluent monolayer	No adherence; Cell clumping; Few spread Cells	Confluent monolayer	No adherence; Cell clumping; Few spread Cells	No adherence; No clumping; Few spread Cells
% viability	100	100	<1	95	<3	60

Treatments A, B, and D showed essentially the same results, with most of the HFF showing normal spreading and viability.

Free PLL was found to be toxic at the concentrations used (treatments C and E). Essentially all cells subjected to treatments C and E took up TB and did not spread on the tissue culture substrate. The cells subjected to treatments C and E also showed extensive aggregation.

Free PEG had no appreciable effect on cell function (treatment D). PEG also had no appreciable ameliorating effect in conjunction with PLL (treatment E).

Incubation with the graft PLL-PEG copolymer of the present invention (treatment F) however, had a remarkable effect on the HFF. In stark contrast to treatment with free PLL, treatment with the copolymer PLL-PEG (at 3 times higher concentration than used for treatments C and E) produced cells that showed no adherence to the substrate, no aggregation in suspension, but a high percent viability. This viability was maintained for well over 24 hours with the HFF still in suspension. This behavior is quite unusual for anchorage dependent cells.

A distinct morphological difference in cells treated with PLL and PLL-PEG was evident. PLL treated cells in suspension showed a rough or ragged surface while those treated with PLL-PEG copolymer of the present invention are smooth and spherical, much like freshly trypsinized cells.

These results indicate that treatment with the PLL-PEG copolymer of the invention is noncytotoxic to HFF. In addition, interaction of the PEG-grafted polycation with the exterior of the cell prevents the cell from adhering to

a substrate. Thus the cytotoxicity of PLL is markedly reduced by PEG grafting.

Five days after the initial treatment, a few of the cells treated with PLL-PEG copolymer begin to show some spreading on the surface of the culture dish. This observation implies that the PLL-PEG copolymer may either have desorbed from the cell surfaces, or cell division may have occurred (which would dilute the concentration of PLL-PEG copolymer on the cell membrane).

EXAMPLE III

Assessment of Efficacy of PLL and PLL-PEG Treatments at Various Dilutions

A similar experiment as outlined in Example II was conducted to test the effects of PLL and PLL-PEG copolymer at various dilutions. Solutions C and F were serially diluted with 10 mM HEPES buffered saline (HBS) to 1/5, 1/25, and 1/125 of their original concentrations, and human foreskin fibroblasts (HFF) incubated in these solutions for 10 minutes. Additional treatments included PEG 20M (a PEG composition having a molecular weight of about 20,000, comprised of two lower molecular weight PEGs (one having a MW ~8,000 and the other having a MW of ~10,000) linked together by a hydrophobic, bifunctional bisphenol-epichlorohydrin linker; available from Union Carbide, Danbury, CT) and PEG 20,000 (a substantially linear PEG having a molecular weight of ~20,000; available from Fluka, Ronkonkoma, NY) at 0.5% in HBS. A control treatment with fibroblast culture media was also run. Results are summarized in Table II, below.

In the Table, the following abbreviations are used:

"adh." for adhesion,
"aggreg." for aggregated, and
"subst." is the abbreviation for substrate.

P-0 refers to cells treated with 0.1% of PLL, and P-5, P-25 and P-125 refer to cells treated with 1/5, 1/25, and 1/125 dilutions thereof, respectively. Similarly, G-0 refers to cells treated with 0.3% of PLL-PEG copolymer, and G-5, G-25 and G-125 refer to cells treated with 1/5, 1/25, and 1/125 dilutions thereof, respectively.

TABLE II

HFF TREATMENT SOLUTIONS

TIME AFTER SEEDING	Control			PLL treatment				PLL-PEG copolymer treatment			
	No PEG	20M PEG	20,000 PEG	P-0	P-5	P-25	P-125	G-0	G-5	G-25	G-125
0 hr	------Minor adh.------ to substr.			Clumped	Clumped	Clumped	Clumped; adh. to subst.	No adh.	No adh.	Minor adh. to subst.	Minor adh. to subst.
3 hr	---------100%--------- adh. to subst.			Clumped; aggreg.; no adh. to subst.	Clumped; aggreg.; no adh. to subst.	Clumped; 5-10% adhered to subst.	Clumped; 5-10% adhered to subst.	No adh.	50% adh.	75% adh. to subst.	100% adh. to subst.
24 hr	---------100%--------- adhesion			No adh; Extensive clumping	No adh; Extensive clumping	Clumped; ~10% adh. to subst.	Clumped; ~10% adh. to subst.	No adh.; no clumping	~60% adh.;	~80% adh.	~100% adhesion
% viability	---------100%---------			<10%	<10%	<10%	<10%	>70%	>70%	>70%	>70%

Observation of the cells immediately after seeding showed all PLL treatments (abbreviated P) to cause clumping of cells. A small number of cells showed adherence in the P-125 treatment. The graft copolymer (PLL-PEG, abbreviated G) treatment showed a decrease in efficacy at the lower concentrations. At dilutions of 25 and 125 (G-25 and G-125), adherence of cells was noted, though not quantified. Treatments with the PEG 20M and PEG 20,000 showed no appreciable difference from the control.

Three hours following the initial seeding, the following observations were made. The PLL treated cells P-0 (0.1% PLL) and P-5 were clumped and aggregated, with none of the cells showing adherence to the substrate. P-25 and P-125 also showed clumping, but approximately 5-10% of cells adhered to the substrate, indicating PLL cytotoxicity at very low concentrations.

Cells treated with PLL-PEG showed an increased adhering tendency with increasing dilutions. G-0 (0.3% PLL-PEG) showed no adhesion and individual free-floating cells. G-5, G-25, and G-125 showed approximately 50%, 75% and 100% adherence, respectively, at 3 hours. G-125 was very similar to the PEGs and the control.

After 24 hours, P-0 and P-5 showed no adherence to substrate, and extensive clumping. P-25 and P-125 also showed clumping, but approximately 10% of the cells were adhered to the substrate, indicating a lower level of toxicity for P-25 and P-125, compared to the higher concentrations used in samples P-0 and P-5.

After 24 hours, G-0 showed no adherence to substrate and no clumping; while G-5, G-25 and G-125 showed increasing levels of adherence of approximately 60%, 80% and 100%, respectively. The PEG treatments and the control

were also 100% adhered.

TB staining at 24 hours showed all PLL treatments to have less than 10% viability, while the treatments with PLL-PEG copolymer showed a viability of greater than 70%. Thus the attachment of PEG to PLL substantially alleviates the PLL toxicity; this effect is apparent at very low concentrations (P-125 = 0.0008% PLL; G-125 = 0.0024%).

EXAMPLE IV

Effect of PLL and PLL-PEG on Confluent Monolayers of Fibroblasts

In order to assess, in a more realistic (although *in vitro* situation), the effects of PLL and PLL-PEG copolymer of the invention on cells which would normally be present in a flattened spread morphology (and not in a rounded morphology), confluent monolayers of HFF were treated with solutions P-0, P-5, G-0, G-5, PEG 20M, and a control (fibroblast culture medium). The cells were exposed to these solutions for 10 minutes, followed by a rinse with HBS, then fibroblast culture medium was returned to the culture dishes.

Short-term observation 15 minutes after treatment showed the P-0 treated cells sloughing off the culture substrate, with approximately 90% of all cells in suspension at 20 minutes.

About 2-5% of cells treated with P-5 were detached from the surface within the same 15 minute period.

HFF treated with solutions G-0, G-5 and PEG 20M showed no appreciable difference from the control cells.

These results indicate that PLL (at 0.1%) is clearly toxic to HFF, while similar concentrations of PLL

modified with PEG show no harmful effects to confluent monolayers of cells. It is noteworthy that the P-5 treatment showed only mild toxicity to spread, confluent fibroblasts, indicating that they may be less susceptible to toxic macromolecules in this state rather than in suspension.

EXAMPLE V

Reversal of PLL-PEG Binding to Cells with Polyanions

It was possible, by addition of heparin sulfate or chondroitin sulfate, to reverse the effect of PLL and PLL-PEG on HFF. Thus, addition of 2.5 U/ml of heparin to the fibroblast culture medium soon after treatment with PLL caused disaggregation of the HFF clumps and resulted in cells that were able to adhere to tissue culture substrates. If, however, the addition of heparin was postponed until several hours after the PLL treatment, reversibility was not possible because the cells had succumbed to PLL toxicity.

This however, was not the case with the PLL-PEG copolymer if the present invention. The nonadhesive, nonaggregating nature conferred upon the fibroblasts by treatment with PLL-PEG copolymer was found to be reversible at least 48 hours after the initial treatment, clearly indicating that these anchorage dependent cells were still alive, despite the fact that they were not adhered to a substrate.

EXAMPLE VI

Resistance of PLL-PEG Treated Cells to Specific Antibodies as Indicators of Conferred Immune Protection

Fibroblasts have receptors for the protein vitronectin on their surfaces. Vitronectin is a cell adhesion molecule (CAM). This receptor (called αV-ß3) can

be targeted with an antibody, anti αV-β3, a rabbit polyclonal. A fluorescently conjugated secondary antibody to anti αV-β3 (e.g., rhodamine conjugated anti IgG, goat anti-rabbit) would permit the visualization of these receptors on the cell surface.

Untreated HFF, PLL treated HFF, and PLL-PEG treated HFF were incubated with anti αV-β3 polyclonal antibody, followed by incubation with the secondary antibody, then observed at the appropriate excitation wavelengths under a microscope. It was observed that the untreated and PLL treated cells showed strong fluorescence, while the PLL-PEG treated cells fluoresced at a much lower level. This observation indicates that the approach of the antibody to the cell is hindered by the presence of PEG.

PLL by itself was found not to affect the receptor-ligand interaction.

Based on the above-described experiments, it is likely that the prevention of protein binding to these cells will render them immunologically unrecognizable.

EXAMPLE VII

Transplantation of PLL-PEG Treated Allogeneic Islets in Rats as a Model for Immunoprotectivity

Rat islets were isolated employing techniques known in the art [see, for example, Lacy and Kostianovsky in Diabetes 16:35 (1967)]. The isolated islets were treated with 0.3% PLL-PEG as described above (see Example II), and transplanted by injection into the peritoneal cavity of diabetic rats. Diabetes was induced by treatment with streptozotocin. Controls were injected with untreated rat islets. Blood glucose levels of these rats were monitored at first on an hourly basis, and then on a daily basis for several weeks. It was found that the control

rats had a reversal of diabetes (indicated by normal glucose levels) for 3-4 days following which the graft failed due to rejection. On the other hand, the rats injected with the PLL-PEG copolymer treated islets showed a continuous reversal of diabetes for several weeks, indicating that the treatment of these cells with PLL-PEG copolymer was effective in immunoprotecting the islets.

EXAMPLE VIII

Crosslinkable Graft Copolymers

A variation on the above theme for the surface treatment of cells is one in which the PLL-PEG graft copolymer has on its structure polymerizable groups such as the acrylate group. The presence of this group on the graft copolymer facilitates polymerization or crosslinking following the absorption of the copolymer onto the cell surface through ionic interactions. The resultant covalently crosslinked network is significantly more stable than the ionically attached graft copolymer. Thus the immunoprotective properties conferred upon the cell by absorption of PLL-PEG on its surface are no longer transient as may be expected through an ionic interaction, but are permanent due to the formation of intermolecular and intramolecular covalent crosslinks formed with the PLL-PEG.

Synthesis of these polymerizable copolymers could have two possible strategies. One involves the synthesis of a PEG that is heterobifunctional, i.e., one end is functionalized with CDI (1,1-carbonyldiimidazole; or other electrophilic derivative) and the other with acryloyl chloride (the reaction of PEG with acryloyl chloride is described below). This technique allows the synthesis of a PLL-PEG graft copolymer in which the free end of PEG contains a polymerizable double bond. The second strategy involves the preparation of PLL-PEG as described above, and

the subsequent reaction of the copolymer with acryloyl chloride to add polymerizable groups. In this case the addition of polymerizable groups to the copolymer is nonspecific, i.e., the substitution occurs on the free end of the PEG as well as on the amines on polylysine.

The reaction of PEG with acryloyl chloride proceeds to completion in about 24 hours when carried out at 50°C. For example, mono-CDI functionalized PEG (i.e., CDI-PEG-OH, prepared as described in Example I) was reacted with an equimolar amount of acryloyl chloride in dry dichloromethane solvent. The reaction was carried out in a round-bottom flask under an inert atmosphere at constant reflux for 24 hours. The resulting product was purified by precipitation with diethyl ether, then dried in a vacuum oven.

Alternatively, PLL-PEG could be treated with acryloyl chloride. In this situation, acrylate substitution would occur on both the PEG chains and the PLL backbone (via the amine groups thereof).

Photopolymerization is the method of choice for covalent crosslinking of the graft copolymer following attachment to the cell surface. Following attachment of the graft copolymer to the cell surface the treated cells are transferred to a physiological buffer solution containing ethyl eosin (EE, 0.1 μM to 0.1 mM), triethanolamine (TEA, 0.1 mM to 0.1 M), and optionally a comonomer (e.g. 1-vinyl 2-pyrrolidinone (VP) at a concentration in the range of about 0.001 to 1.0%, when used). This solution containing islets is well mixed and exposed to a mercury lamp (100 watt) with a bandpass filter (500-560 nm) for approximately 3 minutes. This causes crosslinking of the copolymer on the surface of the cell resulting in the immunoprotective layer. The cells are then transferred to culture.

An alternative technique involves the incubation of the copolymer treated cells with a solution of EE (0.1 μM to 0.1 mM) in physiological buffer for approximately two minutes. In this step the EE complexes with the positively charged polycation on the cell surface. After rinsing in buffer the cells are transferred to a physiological buffer solution containing TEA (conc. as above), and a comonomer e.g. VP (optional). This solution containing islets is well mixed, polymerized as before, and transferred to culture.

EXAMPLE IX

PLL-PEG Solutions in Organ Preservation Media

As noted above, PEG 20M has been used in the preservation of organs. The basis of its activity, though not clearly understood, is believed to be the binding of PEG to cell surface molecules through nonspecific hydrophobic interactions. The PLL-PEG copolymer of the present invention, however, interacts directly through ionic interactions with cell-surface moieties bearing a negative charge. Thus, tissues and organs may be flushed with a solution containing the PLL-PEG copolymer prior to transplantation to, in effect, 'coat' the tissues with PEG, thereby providing an immunoprotective and organ-protective effect.

EXAMPLE X

Stabilization of Liposomes with PLL-PEG for Longer Circulation Times and Increased Biocompatibility

Lipid vesicles or liposomes have been investigated extensively as systems for drug delivery (Gregoriadis, 1987). The commonly used phospholipids that comprise liposomes, such as phosphatidyl choline, phosphatidyl serine, dilaurylphosphatidic acid, and phosphatidylglycerol are negatively charged at

physiological pH. The interaction of polycations such as PLL with the negatively charged phospholipids has been studied quite extensively with regard to conformational changes induced in PLL and consequent stability [Fukushima et al., *Biophysical Chemistry* 34:83 (1989); Houbre et al., *Biophysical Chemistry* 30:245 (1988)]. Stability of liposomes in physiological conditions is a major focus of researchers investigating drug delivery. Although PLL may be used to stabilize lysosomes *in vitro*, PLL coated liposomes *in vivo* are likely to be rapidly overgrown or ingested by macrophages due to the adhesive nature of PLL, thus making them ineffective for the controlled release of drugs. In addition, liposomes may also be destroyed by uptake by the reticuloendothelial system. The addition of the graft copolymers of the present invention to the surface of the liposome is likely to prevent this uptake.

The replacement of PLL by the PLL-PEG copolymer of the present invention, however, promises to provide a liposome that is stable not only due to interactions between negatively charged phospholipid and positively charged PLL, but also because the PLL-PEG copolymer will prevent interactions with proteins, and therefore prevent interactions with cells such as macrophages. This should result in liposomes with long circulation times which can therefore deliver drugs in a controlled fashion.

EXAMPLE XI

Patterned Surfaces for Neural Networks

Investigators in neurology have tried to generate *in vitro* networks of neurons on culture dishes. A problem has been to generate patterned surfaces that are preferentially adherent to cells in order to design 'biological circuits.' By creating a mask of the pattern desired, and applying it to the culture substrate, followed by treatment of the surface with PLL-PEG copolymer, one can

selectively leave the desired pattern adhesive to cells, while the rest of the available surface is rendered nonadhesive to cells.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A method to render cells non-adhesive, said method comprising contacting said cells with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

2. A method according to claim 1 wherein said water-soluble polymer is selected from polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly(hydroxyethyl methacrylate) (pHEMA), polyacrylic acid (PAA), poly(acrylamide), poly(vinyl pyrrolidone) (PVP), poly(ethyl oxazoline) (PEOX), polysaccharides, or copolymers of any two or more thereof.

3. A method according to claim 1 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

4. A method according to claim 3 wherein said water-soluble polymer is polyethylene glycol.

5. A method according to claim 1 wherein either the water-soluble polymer, or the polycationic species, or both contain at least one functional group which is susceptible to free radical polymerization.

6. A method according to claim 5 wherein said composition is further subjected to free radical polymerization conditions.

7. A method according to claim 1 wherein said polycationic species is selected from:

polyethyleneimine, polyallylamine, polyetheramine, polyvinylpyridine,

polysaccharides having a positively charged functionality thereon,

polyamino acids selected from:
poly-L-histidine, poly-im-benzyl-L-histidine,
poly-D-lysine, poly-DL-lysine, poly-L-lysine,
poly-ε-CBZ-D-lysine, poly-ε-CBZ-DL-lysine,
poly-ε-CBZ-L-lysine,

poly-DL-ornithine, poly-L-ornithine,
poly-δ-CBZ-DL-ornithine,

poly-L-arginine,

poly-DL-alanine-poly-L-lysine;

poly(-L-histidine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

poly(L-phenylalanine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine; or

poly(L-tyrosine, L-glutamic acid)-poly-DL-alanine-poly-L-lysine;

random copolymers of:
L-arginine with tryptophan, tyrosine, or serine;
D-glutamic acid with D-lysine; or
L-glutamic acid with lysine, ornithine, or mixtures thereof;

as well as mixtures of any two or more thereof.

8. A method according to claim 1 wherein said polycationic species is selected from polylysine or polyornithine.

9. A method according to claim 1 wherein said cells to be rendered non-adhesive are selected from islets, thyroid cells, adrenal cells, dopamine secreting cells, hepatocytes, or human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV.

10. The cellular product obtained by the method of claim 1.

11. A process to remove composition(s) comprising a polycationic species having water-soluble polymer chains grafted thereon from cells treated in accordance with the method of claim 1, said process comprising contacting said treated cells with an effective amount of an anionic species.

12. A process according to claim 11 wherein said anionic species is monomeric or polymeric.

13. A process according to claim 12 wherein said anionic species is a polyionic species selected from heparin, heparin sulfate, chondroitin sulfate, bovine serum albumin, soluble alginates, hyaluronic acid, pectin, carageenan, or oxidized cellulose.

14. Cells having a modified cell surface which is non-adhesive with respect to mediators of immune response, wherein the surface of said cells have been modified with a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

15. A method to render cells non-immunogenic, said method comprising contacting said cells with a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

16. A method according to claim 15 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

17. A method according to claim 16 wherein said water-soluble polymer is polyethylene glycol.

18. A method according to claim 15 wherein said cells to be rendered non-immunogenic are selected from islets, thyroid cells, adrenal cells, dopamine secreting cells, hepatocytes, or human T-lymphoblastoid cells sensitive to the cytopathic effects of HIV.

19. The cellular product obtained by the method of claim 15.

20. A process to remove composition comprising polycationic species having water-soluble polymer chains grafted thereon from cells treated in accordance with the method of claim 15, said process comprising contacting said treated cells with an effective amount of an anionic species.

21. A method to preserve cells and/or tissues which are subjected to long periods of storage before being used for therapeutic applications, said method comprising contacting said cells and/or tissues with an effective amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

22. A method for associating water-soluble polymer with a cell surface, said method comprising:

grafting water-soluble polymer onto a polycationic resin to produce a copolymer of said water-soluble polymer and said polycation, and thereafter

contacting said cell surface with an effective amount of said copolymer.

23. A method according to claim 22 wherein said copolymer comprises a polycation having grafted thereon at least one water-soluble polymer chain per chain of said polycationic resin.

24. A method for the stabilization of liposomes having negatively charged surfaces, said method comprising contacting said liposomes with an effective, stabilizing amount of a composition comprising a polycationic species having water-soluble polymer chains grafted thereon.

25. A method according to claim 24 wherein said polycationic species has grafted thereon at least one water-soluble polymer chain per chain of said polycationic species.

26. A method for producing neural networks on a substrate, said method comprising:

masking that portion of said substrate which defines the desired network,

rendering the unmasked portion of said substrate non-adhesive by the method of claim 1,

removing the mask, then

allowing cells to spread and grow on said substrate.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 1/02; C12P 1/00; C12N 5/00
US CL :435/1, 41, 240.1, 240.22, 962

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1, 41, 240.1, 240.22, 962

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 91/07951 (Skjak-Braek et al.) 13 June 1991, see page 3 and claim 10.	1-10
Y	Neuroscience Letters, Volume 49, issued 1984, U. T. Ruegg et al., "Growth of Dissociated Neurons in Culture Dishes Coated with Synthetic Polymeric Amines," pages 319-324, see page 319.	1-10
Y	Transplantation, Volume 51, No. 1, issued January 1991, Tianli Zheng et al., "Prolonged Pancrease Preservation Using A Simplfied UW Solution Containing Polytheylene Glycol", pages 63-66, see page 63, abstract.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1993	08 JUN 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer RALPH GITOMER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biomedical Materials Research, Volume 25, issued 1991, Neil P. Desai et al., "Biological Responses to Polyethylene Oxide Modified Polyethylene Terephthalate Surfaces",pages 829-843, see page 829, abstract.	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02609

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Form PCT/ISA/206 Previously Mailed.)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/US93/02609

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-10, drawn to a method of making non-adhesive cells and the product of the method.
II. Claims 11-13, drawn to a process to remove compositions.
III. Claim 14, drawn to cells with modified immune response.
IV. Claims 15-19, drawn to a process to make cells non-immunogenic and the product of the process.
V. Claim 20, drawn to a process to reverse non-immunogenic cells.
VI. Claim 21, drawn to a method of storage.
VII. Claims 22-23, drawn to a method of applying.
VIII. Claims 24-25, drawn to a method of stabilizing liposomes.
IX. Claim 26, drawn to a method for making neural networks.

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(54) Title: PEGYLATION PROCESS

(57) Abstract

The present invention relates to the attachment of a polyethylene glycol (PEG) moiety to a target substrate. Processes for such attachment will be hereinafter referred to as "PEGylation" of the substrate. In particular, the present invention relates to a process for direct covalent PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group in the PEGylation reaction.

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PEGYLATION PROCESS

The present invention relates to the attachment of a polyethylene glycol (PEG) moiety to a target substrate. Processes for such attachment will be hereinafter referred to as "PEGylation" of the substrate. In particular, the present invention relates to a process for direct covalent PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group in the PEGylation reaction.

Covalent attachment of PEG to molecules such as proteins or structures such as liposomes is well known to improve their pharmacological and physiological properties.

EP-A-354855 describes a liposome which comprises a PEG-bound phospholipid wherein the PEG moiety is bonded to a phospholipid present in the liposome membrane. This is claimed to provide a reduction in the absorption of proteins to the liposome *in vivo* and hence an increase in its *in vivo* stability.

EP-A-154316 describes a method for chemically modifying lymphokines by attachment of a PEG moiety wherein the PEG is bonded to at least one primary amino group of the lymphokine. This is claimed to result in the delayed clearance of lymphokines when used as drugs and to decrease their antigenicity.

There are many methods for achieving covalent coupling of PEG to substrates. All such methods require the activation of the PEG by attachment of a group usually referred to as an "activating moiety" or by converting a terminal moiety of the PEG into an activating moiety. This is followed by a second step where the PEG couples to the target molecule, usually via a residual portion of the activating moiety which may be referred to as the "coupling moiety".

Examples of known techniques include:

Succinimidyl Active Ester Methods: see e.g. US Patent 4,412,989; WO 86/04145; WO 87/00056; EP-A-0 247 860, C. Monfardini, O. Shiavon, P. Caliceti, M. Morpurgo, J. M. Harris, and F. M. Veronese, "A branched monomethoxypoly(ethylene glycol) for protein modification," Bioconjugate Chem., 6,62-69 (1995), Zalipsky, S. et al. (1991) in "Polymeric Drugs and Drug Delivery Systems" (R. L. Dunn & R. M. Ottenbrite, eds.) ACS, Washington, DC, Chapter 10, Zalipsky, S. et al. (1992) Biotechnol. Appl. Biochem. **15**:100, Chiu, H.-C. et al. (1993) Bioconjugate Chem. **4**:290, Sirokman, G. & Fasman, G. (1993) Protein Sci. **2**:1161, Veronese, F. M. et al (1989) J. Controlled Release **10**:145, Abuchowski, A. et al (1984) Cancer Biochem. Biophys. **7**:175, Joppich, M. & Luisi, P.L. (1979) Macromol. Chem. **180**:1381, Klibanov, A. L. et al (1990) FEBS Letters **268**:235, Sartore, L. et al (1991) Appl. Biochem. Biotech. **31**:213

Carbonyldiimidazole Method: see e.g. EP-A-0 154 432.

Phenylchloroformate Methods: see e.g. WO 89/06546 and WO 90/15628.

PEG-Succinate Mixed Anhydride Methods: see e.g. Ahlstedt et al (1983) Int. Arch. Allergy Appl. Immunol., 71,228-232; Richter and Akerblom (1983) Int. Arch. Allergy Appl. Immunol, 70, 124-131;

Organic Sulphonyl Halide Methods: see e.g. US Patent 4,415,665.

PEG-Maleimide and Related Methods: see e.g. Goodson & Katre (1990) Biotechnology, 8, 343-346.

Phenylglyoxal Method: see e.g. EP-A-0 340 741

Succinimide Carbonate Method: see e.g. WO 90/13540; WO 91/07190

Cyanogen Bromide Method: see USP 4,301,144

Poly-PEG Maleic Acid Anhydride Method: Yoshimoto et al (1987) Biochem. and Biophy. Res. Commun. 148, 876-882.

Cyanuric chloride method: Abuchowski, A. van Es, T., Palczuk, N.C., & David, F.F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J. Biol. Chem., 252, 3578-3581.

PEG acetaldehyde methods: Royer, G.P. US 4,002,531 EP-A-0154316. Harris, J. M., Yoshinaga, K. Paley, M.S., & Herati, M. R.

(1989). New activated PEG derivatives for affinity partitioning. In D. Fisher & I.A. Sutherland (Eds) Separations Using Aqueous Phase Systems. Applications in Cell Biology and Biotechnology (pp. 203-210). London Plenum Press.

Amine acylation methods (both PEG-COOH and PEG-NH_2): see e.g. EP0072111 and EP 0401384.

Vinylsulfone method: M. Morpurgo, F. M. Veronese, D. Kachensky and J. M. Harris, J. Bioconj. Chem., 7, 363-368 (1996).

PEG epoxide methods: Elling, L. & Kula, M-R. (1991) Biotech. Appl. Biochem. 13,354.

PEG isocyanate method: R. B. Greenwald. A. Pendri and D. Bolikal, J. Org. Chem., 60, 331-336 (1995).

PEG orthopyridyl-disulphide: C. Woghiren, B. Sharma and S. Stein, Bioconj. Chem., 4,314 (1993).

PEG-proprionaldehyde: Harris, J.M., Dust, J.M., McGill, Harris, P.A., Edgell, M.J., Sedaghat-Herati, R.M., Karr, L.J., & Donnelly, D.L. (1991). New polyethylene glycols for biomedical applications. Chapter 27 in S.W. Shalaby, C. L. McCormick, & G. B. Butler (Eds.), Water-Soluble Polymers Washington D.C.: American Chemical Society.

These methods suffer from one or more of the following defects: Substantial loss of biological activity (e.g. 20-95% loss of bio-activity) is frequently seen with the cyanuric chloride method:

Savoca KV, Abuchowski A, van Es T, Davis FF, Palczuk NC (1979), Biochem Biophys Acta 578: 47-53, Ashihara Y, Kono T, Yamazaki S, Inada Y (1978) Biochem Biophys Res Commun **83**:385-391, Kamisaki Y, Wada H, Yagura T, Matsushima A, Inada Y (1981) J Pharmacol Exp Ther **216**: 410-414, Wieder K. J. Palczuk NC, van Es T, Davis F F (1979) J Biol Chem **254**:12579-12587, Nishimura H, Matsushima A, Inada Y (1981) Enzyme **26**:49-53 and Pyatak PS, Abuchowski A, Davis FF (1980) Res Commun Chem Pathol Pharmacol **29**:113-127

The coupling of PEG (or other polymers) to proteins (or other target molecules) is, with few exceptions, in a manner which leaves part of the activating moiety, a coupling moiety, between the PEG and the target molecule. Of the above methods, only the organic sulphonyl halide methods and PEG-acetaldehyde methods disclosed in Royer US 4002531 (1977) and Harris (1989, ibid) couple PEG directly without coupling moieties i.e. to produce a "linkerless" PEGylated product. With the exception of some other PEG acetaldehyde methods where the coupling moiety is ethylene oxide (and thus indistinguishable from PEG itself) and the direct coupling methods above, all other coupling methods incorporate a coupling moiety distinct from the polymer and the target and are thus regarded as "indirect" coupling methods.

The incorporation of a coupling moiety generates further problems depending on the nature of the coupling moiety, thus

(i) some coupling moieties provide targets for enzymatic cleavage or hydrolysis (see below);

(ii) some coupling moieties provide an immunogenic/antigenic group (e.g. the triazine ring of the cyanuric chloride method or the succinyl group of the succinimidyl succinate method and PEG succinate mixed anhydride method);

(iii) some coupling moieties are potentially toxic or are themselves of unknown toxicity but derived from a compound known to be toxic (e.g. the triazine ring of the cyanuric chloride method and reagents in the phenylchloroformate method); and

(iv) some coupling moieties provide reactive groups capable of linking further molecules to the PEG-target construct via the coupling moiety (e.g. the triazine ring of the cyanuric chloride method, Leonard, M. et al., Tetrahedron, 40: 1585 (1984)) and,

(v) Some coupling groups alter surface charge at the site of attachment of the polymer.

Coupling in some instances is thus via an unstable bond liable to be cleaved by enzymes present in serum, plasma, cells or other biological materials or by procedures applied to the PEG-target product. This has two possible deleterious consequences,

(i) the PEG-target construct is degraded enzymatically or by the conditions required for subsequent reaction steps; the former occurs particularly with methods generating ester bonds and probably also with amide bonds; and

(ii) removal of the PEG moiety alters the target molecule; this occurs with some succinimidyl active ester and mixed anhydride methods,

and either or both of these can occur.

Many of the above methods recommend long coupling times and/or a non physiological pH for the PEGylation reaction, thus rendering some target molecules less active or inactive (cf. the cyanuric chloride, phenylchloroformate, acetaldehyde and propionaldehyde methods).

Many of these methods use activated PEG species and/or produce co-products which are toxic in a wide range of bioassays and which are potentially toxic in vivo if not separated from the product (e.g. the phenylchloroformate, cyanuric chloride methods).

Some methods are unsuitable for use in aqueous solution, thus limiting the target molecules to those which will tolerate non-aqueous conditions (cf. the organic sulphonyl halide method using trifluoromethanesulphonyl chloride).

Some of the activated PEG-target constructs are unstable, for instance being subject to hydrolysis during either the activation or coupling reactions (cf. the phenylchloroformate method). For example, PEG acetaldehyde is

sensitive to decomposition under basic conditions and can give inconsistent results.

Ouchi T., et al [(1987) J. Macromol. Sci. Chem. A24 1011-1032] discusses the PEGylation of 5-fluorouracil with various methoxy-PEG derivatives to generate methoxy-PEG ether, ester or amide-linked constructs. The preparation of methoxy-PEG-ether-5-fluorouracil from a methoxy-PEG-Br derivative in chlorobenzene using tetra-n-butylammonium bromide as a phase-transfer catalyst is described. None of the methoxy-PEG-ether-5-fluorouracil derivatives thus produced showed bioactivity (i.e. anti-tumour activity).

Zheng Hu et al (1987) Acta Pharmaceutical Sinica, 22 (8) 637 - 640 discusses the synthesis of PEG-estrogen compounds from chlorinated polyethylene in non-aqueous solvents using the Williamson reaction.

Probably the most advantageous PEGylation method employed hitherto is the TMPEG method, mentioned in WOA-90/04606, which comprises activation of monomethoxy PEG ("MPEG") with 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) to produce tresyl MPEG ("TMPEG") which is subsequently reacted with a target protein molecule to produce monomethoxy PEGylated products. At physiological pH the TMPEG method is a "direct" coupling method in that the PEG moiety is coupled directly to the target substrate without a coupling or linker moiety. A similar technique is described in WO 90/04650 for coupling monomethoxy PEG moieties to DNA/protein complexes.

It is also known that the use of TMPEG as the activated PEG for use in PEGylation can, particularly at very high pH, result in the elimination of

HF by an alternate pathway. This alternative elimination pathway may occur for example when reacting TMPEG with a protein and involves the elimination of HF which converts TMPEG into an intermediate alkene followed by hydration and further elimination of HF to create the acyl fluoride which is converted to the α-sulphonate acid via further hydrolysis. The alkene and acyl fluoride MPEG derivatives can react with target molecules to form a sulphonate amide derivative.

It is clearly desirable to develop a functionalised PEG which is simple and cheap to prepare, which can be used to PEGylate a wide range of potential substrates, which generates a linkerless or directly coupled PEGylated substrate, is capable of pegylating a substrate in both aqueous and non-aqueous solvents and which does not result in any of the undesirable side effects listed above. It is also desirable to have a PEGylation process which functions rapidly under physiological conditions since this is critical for retention of biological activity in the PEGylation of many proteins.

Hence there is provided according to the present invention a process for the PEGylation of a substrate comprising the reaction of a halogenated PEG with the substrate wherein the halogen of the halogenated PEG acts as a leaving group and the PEG is bound directly to the substrate.

In a preferred embodiment of the present invention the halogenated PEG for use in the process of the invention is of general formula I or general formula II.

$$X\text{-}PEG\text{-}Y \qquad (I)$$

$$(PEG)_n\text{-}Y_i \qquad (II)$$

wherein:

In formula I PEG is a bivalent group of formula $-(-CH_2CH_2O-)_m-CH_2CH_2-$, where m is equal or greater than 1, derived from a polyethylene glycol;

X is a halogen atom, a blocking group or an activating group capable of coupling the PEG moiety to another moiety; Y is a halogen; n represents the number of PEG termini and n is equal or greater than 2; i is equal or less than n; and i/n PEG termini are substituted by Y in compounds of formula II.

Halogenated PEGs of Formula I may be monofunctional, homobifunctional, or heterobifunctional activated PEGs i.e. a halogenated PEG of formula I may have two terminal halogens (X and Y are halogens which may be the same or different), or when only one terminal halogen is present the other terminal group X may be either a blocking group or an activating group. Halogenated PEGs of Formula II may be of branched, cruciform or stellate structure. In preferred embodiments of the present invention, X is a blocking group selected from methyl, t-butyl and benzyl ethers.

In further preferred embodiments of the present invention, X is an activating group having an atom that is susceptible to nucleophilic attack or is capable of rendering the terminal carbon atom of the PEG susceptible to nucleophilic attack or equivalent alternative substitution and is preferably a sulphonate ester, a substituted triazine, a N-hydroxysucinimide active ester, an anhydride, a substituted phenyl carbonate, oxycarbonylimidazole, a maleimide, an aldehyde, a glyoxal, carboxylate, a vinyl sulphone, an epoxide, an isocyanate, a disulphide, an acrylate, an allyl ether, a silane or a cyanate ester. More preferably X is an activating group selected from

2,2,2-trifluoroethanesulphonate,
pentafluorobenzenesulphonate,
fluorosulphonate,
2,4,5-trifluorobenzenesulphonate,
2,4-difluorobenzenesulphonate,
2-chloro-4-fluorobenzenesulphonate,
3-chloro-4-fluorobenzenesulphonate,
4-amino-3-chlorobenzenesulphonate,
4-amino-3-fluorobenzenesulphonate,
o-trifluoromethylbenzenesuplphonate,
m-trifluoromethylbenzenesulphonate,
p-trifluoromethylbenzenesulphonate,
2-trifluoromethoxybenzenesulphonate,
4-trifluoromethoxybenzenesulphonate,
5-fluoro-2-methylbenzenesulphonate,
4,6-dichlorotriazine,
6-chlorotriazine,
N-hydroxysuccinimidyl succinate,
N-hydroxysuccinimidyl glutarate,
N-hydroxysuccinimidyl succinamide,
N-hydroxysuccinimidylalkanedioicamides,
N-hydroxysuccinimidyl derivatives of carboxymethylated polymers,
succinimidylcarbonate,
N-hydroxysuccinimidyl esters of amino acids,
succinate mixed anhydride,
succinic anydride,
trichlorophenyl carbonate,
nitrophenyl carbonate,
maleimide,

N-substituted maleimide,
acetaldehyde,
propionaldehyde and chemically equivalent sulphur analogues,
glyoxal,
phenylglyoxal,
acrylate,
methacrylate.

Preferred halogens for the groups X and Y include chlorine, bromine and iodine. Chlorine is most preferred.

In the most preferred embodiments of the present invention, the halogenated PEG is one of

- monomethoxy PEG-Cl
- monomethoxy PEG-Br
- monomethoxy PEG-I
- Cl-PEG-Cl
- Br-PEG-Br
- I-PEG-I

Although some halogenated PEGs are known compounds, it is particularly surprising that they have utility as PEG derivatives suitable for direct use in a PEGylation reaction. It was previously believed that halogens would be of vastly inferior reactivity to known leaving groups such as tosylate or tresylate. For example McMurry J. in "Organic Chemistry" 4th Ed. (1996) cites chlorine as having 300 times less reactivity than tosylate as a leaving group. When considering that tresylate is described as having 100 fold greater reactivity than tosylate (March J. Advanced Organic Chemistry Reactions,

Mechanisms and Structure, 4th Ed. [1992]), it may be concluded that chlorine would be expected to have 30,000 fold less reactivity than tresylate.

Halogenated PEGs may be synthesised by methods well known in the art. PEG may be synthesised or purchased commercially and then derivatised with halogen, activating groups or blocking groups, as required, using methods disclosed in e.g. Bayer E. et al, Polymer Bulletin 8,585 - 592 (1982); Zalipsky, S et al, Eur. Polym. J. Vol 19 No. 12 pp 1177-1183 (1983); Buckmann A. F., Morr. M and Johansson G., Makromol. Chem. 182, 1379-1384 (1981); Harris, J.M. J. Macromol. Sci., Rev. Polym. Chem. Phys. C25(3) 325-373 (1985); Harris, J.M., Struck, E. C., Case, M. G., et al. J. Poly. Sci, Poly. Chem. Ed. 22, 341-352 (1984); Zalipsky, S. & Lee, C. in Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical applications (ed Harris, J. M.) 347-370 (Plenum Press, New York, 1992); and as reviewed in Zalipsky S. Bioconjugate Chem. (1995) 6 150-165 where MPEG-Cl was used to prepare activated PEGs which were subsequently linked to substrates.

Multi-halogenated PEGs can be constructed using either naturally branched PEGs, such as the cruciform PEG found in some preparations of high molecular weight PEGs, or from proprietary multibranched PEGs known as "star" PEGs. Derivatisation of the free PEG termini with halogen is achieved as for halogenated PEGs above.

Reaction conditions for the process of the present invention will clearly depend upon the nature of X, Y and of the substrate.

As indicated above, the PEG moieties of the halogenated PEG's used in accordance with the invention, may desirably be derived from commercially available PEGs. These materials are generally characterised by their number

and weight average molecular weight. For example, PEG-5000 is a polyethylene glycol having a number average molecular weight of about 5000. The size of the PEG moiety to be attached to the target substrate will usually be chosen according to the nature of the substrate and how its properties are desired to be modified by the attachment of the PEG moiety. For example, if the target substrate is a liposome for administration to an animal and it is desired to increase the circulation half life of the liposome after administration, a PEG of molecular weight 1000 to 5000 may be selected. It should be noted, however, that the process of the present invention is generally applicable to the attachment of PEG moieties of any size to target substrates.

PEGylated substrates generated according to the present invention particularly include those which do not lose their bioactivity relative to the unPEGylated substrate. Thus PEGylation according to the present invention may maintain or increase the specific activity of a substrate or it may increase the *in vivo* half-life of a substrate which has had its specific activity decreased, maintained or increased by PEGylation. Additionally PEGylation according to the present invention may differentially modify the specific activity of pleiotropic substrates such as certain proteins.

The term "substrate" as used herein is intended to include any molecule, macromolecule or structure which is capable of being covalently attached to a PEG moiety and which thereby may have its chemical, biological, physiological or physical properties modified. It is not intended to encompass molecules which when reacted with halogenated PEG merely produce a further activated PEG derivative which is to be used as an intermediate to couple the PEG moiety to another substrate. The substrate is not a steroid.

Suitable substrates to which PEG can be attached in accordance with the present invention include materials having biological activity which are useful in, for instance diagnosis or therapy and which are all well known to those skilled in the art. They all contain at least one group capable of reacting with the halogenated PEG. Examples of such reactive groups include primary, secondary and tertiary amino groups, thiol groups and aromatic hydroxy groups.

More specifically, substrates for use according to the present invention include proteins, peptides, amino acids and their derivatives such as: antibodies and fragments thereof; cytokines and derivatives or fragments thereof, for example, the interleukins (IL) and especially the IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and IL-12 subtypes thereof; colony stimulating factors, for example granulocyte-macrophage colony stimulating factor, granulocyte-colony stimulating factor (alpha and beta forms), macrophage colony stimulating factor (also known as CSF-1); haemopoietins, for example erythropoietin, haemopoietin-alpha and kit-ligand (also known as stem cell factor or Steel factor); interferons (IFNS), for example IFNalpha, IFNbeta and IFNgamma; growth factors and bifunctional growth modulators, for example epidermal growth factor, platelet derived growth factor, transforming growth factor (alpha and beta forms), amphiregulin, somatomedin-C, bone growth factor, fibroblast growth factors, insulin-like growth factors, heparin binding growth factors and tumour growth factors; differentiation factors and the like, for example macrophage differentiating factor, differentiation inducing factor (DIF) and leukaemia inhibitory factor; activating factors, for example platelet activating factor and macrophage activation factor; coagulation factors such as fibrinolytic/anticoagulant agents including heparin and proteases and their pro-factors, for example clotting factors VII, VIII, IX, X, XI and XII,

antithrombin III, protein C, protein S, streptokinase, urokinase, prourokinase, tissue plasminogen activator, fibrinogen and hirudin; peptide hormones, for example insulin, growth hormone, gonadotrophins, follicle stimulating hormone, leutenising hormone, growth hormone releasing hormone and calcitonin; enzymes such as superoxide dismutase, glucocerebrosidase, asparaginase and adenosine deaminase; vaccines, for example hepatitis-B vaccine, malaria vaccine, melanoma vaccine and HIV-1 vaccine; transcription factors and transcriptional modulators; carbohydrates, glycosoaminoglycans, glycoproteins and polysaccharides; lipids, for example phosphatidyl-ethanolamine, phosphtidylserine and derivatives thereof; sphingosine; and derivatives thereof; nucleotides, nucleosides, heterocyclic bases, DNA, RNA, synthetic and non-synthetic oligonucleotides including those with nuclease resistant backbones; vitamins; antibiotics including lantibiotics; bacteristatic and bactericidal agents; antifungal, anthelminthic and other agents effective against infective agents including unicellular pathogens; small effector molecules such as noradrenalin, alpha adrenergic receptor ligands, dopamine receptor ligands, histamine receptor ligands, GABA/benzodiazepine receptor ligands, serotonin receptor ligands, leukotrienes and triodothyronine; cytotoxic agents such as doxorubicin, methotrexate and derivatives thereof.

The substrate may also be part of a larger multi-molecular structure. These include cells or parts thereof, for instance erythrocytes, erythrocyte "ghosts" and leukocytes, viruses, unicellular organisms, liposomes such as multilamellar vesicles and unilamellar vesicles, micelles and micelle-like structures, and aggregates, microemulsions, coacervates, emulsions and suspensions of the foregoing. The substrate may also be a surface on a device such as a catheter, stent, contact lens or artificial valve.

It will be appreciated that when the substrate is part of such a structure there will generally be many reactive groups in each structure; treatment according to the invention may therefore produce a structure bearing many PEG moieties. When the PEG is bi- or multi-valent, reaction with a multimolecular substrate may result in intermolecular cross-linking by the PEG between molecules of the same target structure and/or between molecules of different target structures as well as intramolecular bonding of the PEG to more than one position on the same molecule of a target structure.

Substrates lacking a reactive group may be modified so as to create one or more reactive groups; this is within the ability of those skilled in the art and can be achieved by well-known techniques.

Some substrates (e.g. RNA and single stranded DNA) pose special problems because they may provide too many reactive groups to which the PEG would attach in a standard reaction. Therefore, if desired, some groups may be temporarily protected by involvement in an appropriate conformation precluding nucleophilic attack on the halogenated PEG, as for example by the hydrogen bonding associated with base pairing of DNA (see below).

The term "blocking group" as used herein is intended to imply a moiety which when covalently bound to a PEG terminus, is capable of preventing the attachment of an activating group to that terminus during the activation process.

An embodiment of the present process involves site-specific modification of DNA, RNA and synthetic oligonucleotide targets (or of any molecule containing and amino or other reactive group which can participate in interactions such as hydrogen bonding with another molecule or compound)

by precluding nucleophilic attack on the halogenated PEG species by reactive groups on the target. The bases adenine (A), cytosine (C) and guanine (G) [but not uracil (U) or thymine (T)] provide suitable targets in DNA, RNA and synthetic oligonucleotides for modification with PEG moieties according to the invention and thus these are special targets with the problem that there may be too many available reactive groups to which the polymer can be attached. By using various restriction fragment DNA cleavage sites as a model system, selected bases A, C or G can be modified by the expedient of leaving short stretches (e.g. 2-4 bases) single stranded. Adenine bases appear to be the most susceptible to such modification. Blunt ended double stranded DNA is not readily coupled under the conditions described, indicating that hydrogen bonding between base pairs is sufficient to preclude interaction of the amino groups of A, C and G bases with the activated PEG.

Site-specific DNA modification by polymer can be achieved by the expedient of including one or more A, C or G bases in a short single stranded section of DNA by appropriate restriction enzyme digestion or by hybridising oligonucleotides of dissimilar lengths with the DNA to protect bases which are not to be modified or by exploiting the natural strand asymmetry of polymerise chain reaction products which have a one base-pair overhand, or by exploiting localised regions of single strandedness achieved by natural or artificial localised melting of the double helix. The reaction of methoxyPEGBr with 5-FU is not included within the present invention.

Also provided according to the present invention are products produced by a process of the present invention; the use of such products in medical therapy; pharmaceutical formulations comprising products of the present invention; the use of a product or a pharmaceutical formulation of the present invention in the manufacture of a medicament for use in medical

therapy; and methods of medical therapy comprising the administration of a product or a pharmaceutical formulation of the present invention, to a patient.

The term "medical therapy" as used herein includes therapeutic, diagnostic and prophylactic regimes.

Non-limiting examples of the invention will now be described with reference to the accompanying Figures, which show:-

FIGURE 1 Reverse phase liquid chromatography elution profiles of a) MPEG-Cl; b) MPEG and c) a mixture of MPEG-Cl and MPEG.

FIGURE 2 a) A representative elution profile of the reaction products of the PEGylation of lysozyme using MPEG-Cl, separated on a Superose 12 column.
b) A further example of a similar experiment to that shown in Figure 2a

FIGURE 3 A representative elution profile of reaction products of the PEGylation of lysozyme using TMPEG, separated on a Superose 12 column.

FIGURE 4 Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme by MPEG-Cl at alkaline pH.

FIGURE 5 Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme by MPEG-Cl at a) 4°C for 21min and b) 4°C for 72.5h.

FIGURE 6 Elution profiles on Superose 12 of reaction products of PEGylation of lysozyme using TMPEG at 3°C, 15.5°C and 22.5°C.

FIGURE 7 Reverse phase liquid chromatography of polymer species (MPEG,MPEG-Cl and TMPEG in peaks 1-3 respectively) formed using the procedure set out in example 5.

FIGURE 8 The elution profile from a Superose 12 column (using a computerised FPLC) of the reaction products of the PEGylation of lysozyme using MPEG-Cl produced by the method of example 5.

FIGURE 9 The elution profile from a Superose 12 column (using a computerised FPLC) when the amount of TMPEG is equivalent to 10% of the total activated polymer used in Figure 8 above (i.e. similar to the level of contaminating TMPEG in the sample used for PEGylation in Figure 8).

FIGURE 10 The elution profile from a Superose 12 column (using a computerised FPLC) of the reaction products of the PEGylation reaction of lysozyme described in Example 6; i.e. using MPEG-C1, produced by the method of Example 5, from which traces of TMPEG had been removed by prolonged hydrolysis as set out in Example 6.

FIGURE 11 Dose response curves of PEGylated GM-CSF produced using MPEG-Cl synthesised as set out in Example 5 and sham-treated

controls exposed to MPEG. The results shown in panels a, b and c are from three independent experiments.

FIGURE 12 Dose response curves of PEGylated EPO produced using MPEG-Cl synthesised as set out in Example 1 and a sham-treated control exposed to MPEG.

FIGURE 13 Dose response curves of PEGylated EPO produced using MPEG-Cl synthesised as set out in Example 5 and sham treated controls exposed to MPEG. Panels a-c show three independent experiments.

FIGURE 14 Acid elimination by MPEG-Cl at pH 7 and Fluoride measurements (for comparison with Figure 15).

FIGURE 15 Fluoride and acid elimination by TMPEG at pH 7: a) TMPEG produced by the method of [WO 95/06058]; b,c) TMPEG produced by two alternate manufacturing procedures.

FIGURE 16 Acid elimination for MPEG-Cl at pH 9 and Fluoride measurements for comparison with Figure 17.

FIGURE 17 Fluoride and acid elimination for TMPEG at pH 9.

EXAMPLES

EXAMPLE 1 Preparation and characterisation of MPEG chloride

MPEG-chloride was synthesised as described by a modification of the method of *Bayer et al (1982)*. Methoxypolyethylene glycol (Molecular weight 5000, Shearwater Polymers Inc) (5g) was refluxed on an oil bath with twice re-distilled thionyl chloride (10ml) for 15h under nitrogen. The thionyl chloride was removed by distillation. 5ml of dry toluene (molecular sieve, 3A, BDH) was added and distilled off. 5ml of dry dichloromethane (molecular sieve, 3A, BDH) was then added and distilled off. The residue was dissolved in 20 ml of dry dichloromethane and 200ml of dry ether was added at room temperature and the mixture stirred in an ice bath. After storage overnight at -20°C, the white solid was removed by filtration, redissolved in 20 ml of dry dichloromethane and reprecipitated with 200ml of dry ether. The precipitate was removed by filtration and dried in vacuo *(Yield 3.8 g)*.

The product showed a single peak on reverse phase liquid chromatography that was distinct from the single peak shown by the MPEG starting material (Figures 1a-c), indicating complete derivatisation of the starting MPEG to MPEG-Cl.

All the samples were analysed as 0.2% w/v solutions in 30% CH_3CN/70%H_2O on a reverse phase column PLRP-S 100A 5µ from Polymer Laboratories, using 30 to 100% CH_3CN gradient. The elution conditions were as follows: using a flow rate of 0.5ml/minute, 30-50% CH_3CN over 20 minutes, then 50-100% CH_3CN over 2 minutes, held at 100% CH_3CN for 3 minutes under isocratic conditions, reverted back to 30% CH_3CN over 1 minute, and finally held at 30% CH_3CN for 5 minutes. The sample was injected via a 20µl loading loop. An evaporative Mass Detector (PL-EMD 960; Polymer Laboratories) at 85°C with gas flow at 5.5litres/minute, was used to monitor the samples.

Figure 1a shows a typical elution profile of MPEG-Cl. A major peak is seen eluting with a retention time at 16.3 min (range 15.9-16.3 min in three experiments). Figure 1b shows a typical elution profile of MPEG-5K, the starting material for preparation of MPEG-Cl). A single peak is seen eluting with a retention time of circa 13.7 min (range 13.0-13.7 in five experiments). Figure 1c shows the elution profile of a mixture of the MPEG-Cl and MPEG-5K samples, by pooling equal volumes of MPEG-Cl and MPEG-5K used to produce the profiles in Figures 1a and 1b above. Two well resolved peaks are seen with retention times corresponding closely to those obtained in Figures 1a and 1b.

1H nmr of the MPEG-Cl in d_6-DMSO showed an absence of -OH signal (which is typically seen around at 4.56ppm for MPEG in DMSO). A complex multiplet centred around 3.7 ppm was consistent with literature values for O-CH2-CH2-Cl.

Example 2 PEGylation of lysozyme

51.25mg of activated MPEG-Cl as prepared in (Example 1) was reacted with 0.466ml of 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at 27°C. The initial polymer concentration was 110 mg/ml. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 50ml of PBS buffer at a flow rate of 0.3ml/min with continuous UV monitoring (214nm) at the outlet. The sensitivity of the UV detector was set at 0.5 absorbance units. The unreacted lysozyme eluted at circa 19.52ml and the PEGylated lysozyme conjugates eluted at circa 10.79, 13.02, 14.94 (Figure 2a). The chromatogram indicates that significant reaction occurred between the MPEG-Cl and lysozyme under the

above conditions. This reactivity rate was reproduced in further independent experiments.

For example, 48.15mg of MPEG-Cl was reacted with 0.438ml of 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at 28°C. The initial polymer concentration was 109 mg/mL. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded in a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted as for the example above except that the sensitivity of the UV detector was set at 1.0 absorbance units. The unreacted lysozyme eluted at circa 19.65ml and the PEGylated lysozyme conjugates are eluted at circa 10.49, 12.70, 14.32, 15.17 (Figure 2b). Again, the chromatogram indicates that significant reaction occurs between the MPEG-Cl and lysozyme under the above conditions.

Comparative example 2: PEGylation of lysozyme with tresyl monomethoxy PEG as the activated polymer

68.3 mg of tresylated MPEG (TMPEG), prepared as previously described [WO 95/06058], was reacted with 0.580 mg of lysozyme in a total volume of 0.580 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23°C. An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 1 minute, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column is eluted with 25 ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The unreacted lysozyme eluted at 19.40 ml and the PEGylated lysozyme conjugates are eluted at circa 12.05, 14.24 and 15.74 ml (Figure 3). From the profile it is evident that the PEGylation reaction is occurring at a

similar rate to that achieved with the MPEG-Cl sample in Example 2, which is surprising, given the anticipated low reactivity of the PEG-Cl.

Example 3 PEGylation of lysozyme at alkaline pH.

One disadvantage of the TMPEG method overcome by the present invention is that with the former, if the activated PEG is exposed to high pH, fluoride elimination occurs. This has two consequences: first the activated polymer is rapidly exhausted and, second, a proportion of the linkages made between the polymer and the target molecule will have an alternate linkage (a sulphonate amide linkage as opposed to a secondary amine linkage). This linkage alters surface charge; introduces a coupling moiety into the product and the coupling between the polymer and target molecule or structure is unstable, particularly at alkaline pH. MPEG-Cl does not break down as rapidly as TMPEG at alkaline pH (see Example 9 below).

50.0 mg of activated MPEG-Cl was reacted with 0.455 ml of 1mg/ml lysozyme (Fluka) in phosphate buffer (pH 8.66) for 21 minutes at 26^0C. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded in a Superose 12 column and eluted as in Example 2. The chromatogram shows that one peak elutes at circa 17.03 ml and other peaks eluted at circa 12.64, 14.32 (largest) (Figure 4). There was no peak at the location of unmodified lysozyme (circa 19.2ml) indicating that the MPEG-Cl and lysozyme react significantly faster under the above conditions. It should be noted that there is no basis for the formation of an alternate linkage with the MPEG-Cl method.

Example 4: PEGylation of lysozyme at 4°C

One further disadvantage of the TMPEG method overcome by the present invention is that, with the former, if the activated PEG is used at low temperature, longer PEGylation times and/or higher polymer concentrations are required to achieve the same degree of PEGylation as achieved at room temperature. With TMPEG, however, the duration of the PEGylation reaction cannot be much prolonged since the activated polymer hydrolyses at a significant rate (see Example 9 below). The ability to PEGylate substrates at low temperatures can be of advantage with target molecules or structures that are unstable at higher temperatures. In addition, polymer concentrations can be lowered significantly if longer reaction times are feasible.

49.80mg of MPEG-Cl was reacted with 0.453mL of 1mg/ml lysozyme (Fluka) in phosphate buffer (20mM, pH 7.0) for 21 minutes at 4°C. The initial polymer concentration was 110 mg/mL. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded on a Superose 12 column and eluted as in Example 2. The unreacted lysozyme eluted at circa 19.55ml and the PEGylated lysozyme conjugates eluted at circa 10.61, 12.61, 14.28, 15.10 (Figure 5a). The chromatogram indicates that although some reaction has occurred between the MPEG-Cl and the lysozyme the proportion of unmodified material is higher than was observed with similar reactions carried out at room temperature (see Figures 2a and 2b).

However, this reaction could be prolonged until essentially all lysozyme had reacted. 47.89mg of MPEG-Cl was reacted with 0.435mL of 1mg/ml lysozyme (Fluka) in phosphate buffer for 72.5 hours at 4°C. As above, The initial lysozyme concentration was 110 mg/ml. 100ul of the reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded in a Superose 12 column and eluted as in Example 2. The unreacted lysozyme was indistinguishable among three fragments at 16.91, 18.02 and 19.31. The

PEGylated lysozyme conjugates eluted at circa 12.72 and 14.86 (Figure 5b). The chromatogram indicates that almost complete reaction has occurred between MPEG-Cl and lysozyme.

Comparative example 4: PEGylation using TMPEG at reduced temperature.

Figure 6 shows the result of reaction of lysozyme with TMPEG at three different reaction temperatures.

135mg of TMPEG-12K was reacted with 0.5ml of 1mg/ml lysozyme (Fluka) in phosphate buffer for 21minutes. The reaction was performed three times at different temparatures: 3°C, 15.5°C and 22.5°C. 100ml of each reaction mixture was diluted with 400ul of PBS buffer and then 200ul was loaded on a Superose 12 column and eluted as in Example 2.

The estimated areas under the curve for the unmodified lysozyme peak were 52.5% at 3°C, 46.5% at 15.5°C and 23% at 22.5°C.

Example 5: **An additional synthetic route for MPEG-Cl**

MPEG-Cl is also produced by variation of the previously reported manufacturing procedure for TMPEG [WO 95/06058]. This product is exposed to more rigorous washing steps than MPEG-Cl derived from the thionyl chloride method and is included here because this may be the basis of the observed superior retention of bioactivity.

MPEG (Mr 5000; 18g; Shearwater Polymers Inc, USA) was dissolved in toluene (40ml) and the water-organic azeotrope was distilled off, followed

by the bulk of the toluene (109-110°C), obtaining about 35 ml of distillate. The remaining toluene was removed by rotary evaporation under reduced pressure.

The dried MPEG was dissolved in a dry acetonitrile (40ml; dried overnight with molecular sieve 3A (3Angstrom), BDH, UK, added at 10g per 50ml) at room temperature and then cooled in a water-ice bath to 1°C and magnetically stirred. One ml of ice-cold pyridine (BDH, UK) was added over 1 min with constant stirring. Tresyl chloride (1ml; Fluka AG, Switzerland) was then added drop-wise to the stirred solution over 5 min. The solution was then placed at room temperature and stirring was continued for a further 2 h. Acetonitrile was then removed under reduced pressure with occasional warming with a 70°C water bath.

The solid product was dissolved in methanol-HCl (300ml; prepared using 0.75ml conc HCl to 2.5l methanol) and cooled to -20°C overnight. The white precipitate was collected by centrifugation at 0°C and redissolved in 200ml of methanol-HCl. The solution was cooled in ice/salt for 30min and the precipitate isolated by centrifugation. To free the sample of pyridine, the process was repeated until the absorbance of the supernatant at 255nm was at a minimum. Typically 12 washes are required and the minimum absorbance (1cm path length) is 0.02, in this instance, the minimum was 0.04 and 14 washes were used without further improvement. The sample was then dissolved in methanol (200ml) and reprecipitated twice before being dried by rotary evaporation, and then overnight in a freeze dryer (yield 16g).

Analysis by reverse phase liquid chromatography was performed as in Example 1 using a Polymer Laboratories PLRP-6 column and a PL- EMD960 mass detector (Figure 7). The product contained negligible amounts of MPEG

(<1%, see peak at 15.0min) and of the activated PEG species 92.6% was MPEG-Cl (peak at 17.7min) and 7.4% was TMPEG (peak at 19.3min) 1H-nmr and 19F-nmr showed the sample to be substantially MPEG-Cl with some TMPEG. Elemental analysis detected chlorine 0.58% (theoretical chlorine content for 100% MPEG-Cl of molecular weight 5K is 0.7%)

Example 5A Preparation of PEG protein conjugates

70.4 mg of MPEG-Cl produced by the method of Example 5 were reacted with 0.640 mg of lysozyme in a total volume of 0.640 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23^{0}C. An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 3 minutes, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 25 ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The unreacted lysozyme is eluted at circa 19.28 ml and the PEGylated lysozyme conjugates eluted at circa 12.38, 14.38, 14.88 and 15.64 ml (Figure8).

Thus reaction with lysozyme (Figure 8) showed only a slightly lower reactivity than the MPEG-Cl prepared as in Example 1 and reacted with lysozyme in Figures 2a and b. That the reactivity was due to the MPEG-Cl and not the contaminating TMPEG was demonstrated in two ways: first by showing that an equivalent amount of TMPEG to that contaminating the MPEG-Cl preparation had a much lower reactivity (comparative Example 5 Figure 9), and second, that after prolonged hydrolysis sufficient to convert the residual TMPEG to MPEG the remaining MPEG-Cl still reacted (Example 6, Figure 10).

Comparative example 5: PEGylation of lysozyme with tresyl monomethoxy PEG as the activated polymer

6.8 mg of tresylated MPEG (92.2% purity, with 8.8% MPEG-C1 and negligible MPEG; assessed by reverse phase chromatography using a Polymer Laboratories PLRP-6 column and a PL-EMD960 mass detector) was reacted with 0.580 mg of lysozyme in a total volume of 0.580 ml of 20mM phosphate buffer, pH 7, for 21 minutes at 23^0C. An aliquot, (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 1 minute, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column is eluted with 25ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The amount of tresylated MPEG used in this reaction was reduced ten fold relative to the MPEG-Cl preparation used in example 5 (i.e. to an amount slightly more than the contaminating TMPEG present in Example 5), however much less PEGylation was observed (Figure 9) than that observed in the MPEG-C1 example (Figure 8), despite the TMPEG exposure being similar. Thus showing that the reaction in Example 5 was due to the MPEG-Cl.

Example 6: Preparation of PEG-protein conjugates after hydrolysis of residual TMPEG in the MPEG-C1 sample of Example 5.

An aliquot of the MPEG-C1 sample (105mg) prepared in Example 5 was subjected to hydrolysis in water (525ul) for 17 days. After this the sample contained 82.4% MPEG-C1, 0% TMPEG and 17.6% MPEG (assessed by reverse phase chromatography using a Polymer Laboratories PLRP-6 column and a PL-EMD960 mass detector).

275 ul of this sample was reacted with 0.5mg of lysozyme in a total volume of 0.5 ml of 20mM phosphate buffer, pH7 for 21 minutes at 23^0C. An aliquot (100ul) of the reaction mixture was diluted with 400ul of PBS and 200ul was loaded, within a further 1-2 minutes, onto a Superose 12 column fitted to a computerised FPLC system from Pharmacia (Sweden). The column was eluted with 25ml of PBS at a flow rate of 0.3 ml/min with continuous UV monitoring (214 nm) at the outlet. The sensitivity of the UV detector was set at 0.2 absorbance units. The unreacted lysozyme eluted at 18.75ml and the PEGylated lysozyme conjugates are eluted as a shouldered peak at circa 14.33ml with two subsidiary shoulders evident at slightly higher elution volumes (Figure 10). From the profile it is evident that the PEGylation reaction is surprisingly, given the theoretically anticipated very low reactivity of MPEG-C1, still occurring at a significant rate despite the absence of any TMPEG and despite conversion of a proportion of the MPEG-Cl to MPEG.

Example 7: PEGylation of GM-CSF and retention of bioactivity

10 ul of GM-CSF (Hoescht) at 10 ug/ml in PBS were mixed with 15 ul of a solution of MPEG-Cl (produced by the method of Example 5) at circa 250 mg/ml and 15 ul of sterile PBS (Gibco) in a sterile eppendorf tube. Sham treatment controls were set up with MPEG-5K obtained from Union Carbide. The reaction mixture was incubated using a rotary mixer for 2 h at room temperature. It has previously been established that, given the reactivity rate of the activated polymer, these reaction conditions produce a statistical mixture of PEGylated GM-CSF products with mainly 1-3 PEG chains per molecule and over 75% modification. 8 ul of reaction mixture were then added to 10 ml of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum, Life Technologies) to obtain a solution of GM-CSF at 2 ng/ml. The bioactivity of the samples was tested in thymidine uptake assays using a GM-

CSF responsive cell line in 96 well microtiter plates (Nunclon). The samples were diluted with fully supplemented RPMI-1640 corrected for PBS content (64 ul of sterile PBS were added to 80 ml of RPMI-1640) in order to obtain a range of concentrations of GM-CSF from 0.05 to 0.5 ng/ml. The 150 ul solution of GM-CSF in each well received $5x10^5$ TF-1 cells (starved for 24h, i.e. grown for 24h without addition of GM-CSF) and the plate was then incubated for 18h at 37°C under 5% CO_2 atmosphere. The growth stimulation is then quantified using ^{3}H-Thymidine incorporation. [^{3}H]-Thymidine stock (Amersham - TRK120 - batch: B395) was 100 fold diluted and 50 ul of this solution were added to each well. The plate was further incubated for 4h at 37°C under 5% CO_2 atmosphere. The cells were harvested onto a glass filter (Wallac, size 90x120mm), the filter was dried for 2h at 75°C and the dried filter was transferred to a bag (Wallac, size 90x120mm) containing 5 ml of scintillation liquid (Wallac, Betaplate Scint.). the beta emission was quantified using a beta counter (Wallac, 1450 Microbeta plus). The data were background subtracted and CPM-background was plotted against GM-CSF concentration (ng/ml). This experiment was repeated 3 times (Figure 11a-c). The incubation of GM-CSF with this batch of MPEG-Cl resulted in a conservation of bioactivity of 52.8% +/- 5.8%.

Example 8 : PEGylation of Erythropoietin (EPO) and retention of bioactivity.

5 ul of EPO (Cilag) at 3200 U/ml in PBS were mixed with 29 ul of an MPEG-Cl solution at circa 250 mg/ml and 61 ul of sterile PBS (Gibco) in a sterile eppendorf tube. The MPEG-Cl was produced by the method of Example 1. Control sham-treated samples were also prepared using MPEG (obtained from Shearwater Polymers Inc.). The reaction mixture was incubated

using a rotary mixer for 2 h at room temperature. 705 ul of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum) (Life Technologies) were then added to 95 ul of reaction mixture to obtain a solution of EPO at 20 U/ml. The bioactivity of the samples was tested in a 96 wells microtiter plate (Nunclon). The samples were diluted with fully supplemented RPMI-1640 corrected for PBS (9.5 ml of sterile PBS were added to 70.5 ml of RPMI-1640) in order to obtain a range of EPO concentrations from 1 to 10 U/ml. The 150 ul solution of EPO in each well received $5x10^5$ TF-1 cells (starved for 24h, i.e. grown for 24h without addition of GM-CSF, the usual cell growth support for this cell line) and the plate was incubated for 18h at 37°C under 5% CO_2 atmosphere. The growth stimulation was then quantified using [^{3}H]-Thymidine incorporation. [^{3}H]-Thymidine stock (Amersham - TRK120 - batch: B395) was 100 fold diluted and 50 ul of this solution were added to each well. The plate was further incubated for 4h at 37°C under 5% CO_2 atmosphere. The cells were harvested onto a glass filter (Wallac, size 90x120mm), the filter was dried for 2h at 75°C and the dried filter was transferred to a bag (Wallac, size 90x120mm) containing 5 ml of scintillation liquid (Wallac, Betaplate Scint.). the beta emission was quantified using a beta counter (Wallac, 1450 Microbeta plus).

The data were background subtracted and CPM-background were plotted against EPO concentration (as U/ml, i.e. without adjusting for loss of native activity; Figure 12). There was no significant loss of bioactivity.

5 ul of EPO (Cilag) at 3200 U/ml in PBS were mixed with 29 ul of an MPEG-Cl solution at circa 250 mg/ml and 61 ul of sterile PBS (Gibco) in a sterile eppendorf tube. The MPEG-Cl was prepared as in Example 5. Sham treated control EPO was also prepared using MPEG-5K obtained from Union Carbide. The reaction mixture incubated using a rotary mixer for 2 h at room

temperature. 705 ul of RPMI-1640 medium (containing 10% of heat inactivated Foetal Calf Serum, Life Technologies) were then added to 95 ul of reaction mixture to obtain a solution of EPO at 20 U/ml. The bioactivity of the samples was tested as described for Figure 12.

This experiment was repeated 3 times. The data were background subtracted and CPM-Background were plotted against EPO concentration (U/ml) (Figure 13a-c). At low doses of the reaction products, the dose response curves were superimposable, but at higher doses in two experiments there was progressive departure between the test and control curves. This indicates the presence of some toxic or inhibitory material (the EPO assay is particularly sensitive to inhibition, much more so than the GM-CSF assay). The level of toxicity observed is lower than for several other PEGylation procedures previously examined (cf. the cyanuric chloride method and the phenylchloroformate method *(Francis, G.E. et al (1996) J. Drug Targeting 3 321-340)*. The superimposition of the upward part of the test and control dose response curves at low doses of test material indicates no significant loss of bioactivity.

Example 9 and comparative Example 9: Breakdown of MPEG-Cl and TMPEG in aqueous solution.

One advantage of the present invention is the relative stability of the activated polymer.

Figure 14 shows the effect for MPEG-Cl and Figure 15 a-c shows the breakdown of three TMPEG samples.

Figures 14 and 15a-c compare the breakdown rates at pH 7 for MPEG-Cl made by the method of example 1 and three TMPEG samples made by different manufacturing techniques.

The release of acid and of fluoride from samples of activated MPEGs at pH 7 and also at pH 9 were measured in a pH-stat (Mettler Toledo DL 77 titrator) fitted with a fluoride electrode (Mettler Toledo DX 219) and pH electrode (Metler Toledo DG101-SC). 25 ml of 0.9% NaCl was adjusted to pH 7 or 9 with approximately 0.01 M NaOH (standardised by potassium hydrogen phthalate titration). MPEG-Cl or TMPEG (approx 100mg; approx 20 umoles) was added to the saline and simultaneous measurements of fluoride concentration and alkali consumed were made at 20 second intervals for up to 60 min. Results are plotted as umoles of fluoride present and acid produced as a function of time. Samples of activated polymers dissolved in the NaCl required NaOH to bring to the starting pH, indicating that they were not neutral but were somewhat acidic. This immediate uptake of alkali was then followed by a steady uptake as acid was released progressively, and is seen as an intercept at zero time in the plot of alkali uptake against time. This value can be subtracted to provide a plot of acid release at the selected pH.

The changes in fluoride in Figure 14 are an artefact of the impact of OH ions on the fluoride electrode.

Figure 15a shows a sample of TMPEG prepared as previously described [WO 95/06058] with very low fluoride elimination. Note that its acid elimination is substantially higher than that of the MPEG-Cl sample. Figures 15b and c show two samples of TMPEG (obtained from Sigma and Shearwater Polymers Inc) made by different manufacturing procedures and

exhibiting higher fluoride elimination. A combination of hydrolysis to MPEG and trifluoroethanesulphonic acid and fluoride elimination thus produces substantially faster breakdown of TMPEG versus MPEG-Cl. Note that MPEG-Cl left in water for 17 days still retained good reactivity (see above), implying that the acid elimination rates at acidic pH is even lower than that at pH 7.

Figures 16 and 17 show comparable break down rates for MPEG-Cl and TMPEG at pH 9.0.

CLAIMS

1. A process for the PEGylation of a substrate, comprising the reaction of a halogenated PEG with the substrate and the PEG is bound directly to the substrate, with the proviso that the substrate is not a steroid or when the halogenated PEG is PEG-bromide the substrate is not 5-fluorouracil.

2. A process according to claim 1 wherein the halogenated PEG is of general formula I or II

$$X\text{-PEG-}Y \quad (I)$$

$$(PEG)_n\text{-}Y_i \quad (II)$$

wherein:

PEG is a bivalent group of formula $-(-CH_2CH_2O-)_m-CH_2CH_2-$, where m is equal or greater than 1, derived from a polyethylene glycol; X is a halogen atom, a blocking group or an activating group capable of coupling the PEG moiety to another moiety; Y is a halogen; n is the number of PEG termini; n is equal or greater than 2; i is equal or less than n and i/n PEG termini are substituted by Y in compounds of Formula II.

3. A process according to claim 2 wherein Y is Cl, Br or I.

4. A process according to either of claims 2 and 3 wherein X is a blocking group and is methyl, t-butyl or benzyl ether.

5. A process according to either of claims 2 and 3, wherein X is a tresyl activating group.

6. A process according either of claims 1 and 2, wherein the halogenated PEG is one of; methoxy-PEG-Cl, methoxy-PEG-Br, methoxy-PEG-I, Cl-PEG-Cl, Br-PEG-Br or I-PEG-I.

7. A process according to either of claims 1 and 2 wherein the substrate is selected from proteins, peptides, DNA, RNA, nucleotides, nucleotide analogues, hormones other than steroids, antibiotics, liposomes, viruses, unicellular organisms, micelles, metallic plastic, or polymeric surfaces.

8. A product produced by a process according to any of claims 1 to 7.

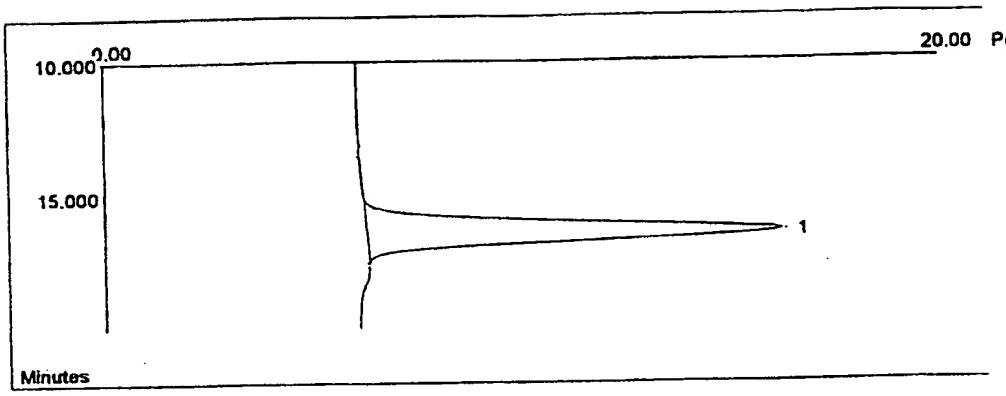
9. A product according to claim 8, for use in medical therapy.

10. A pharmaceutical formulation comprising a product according to claim 8.

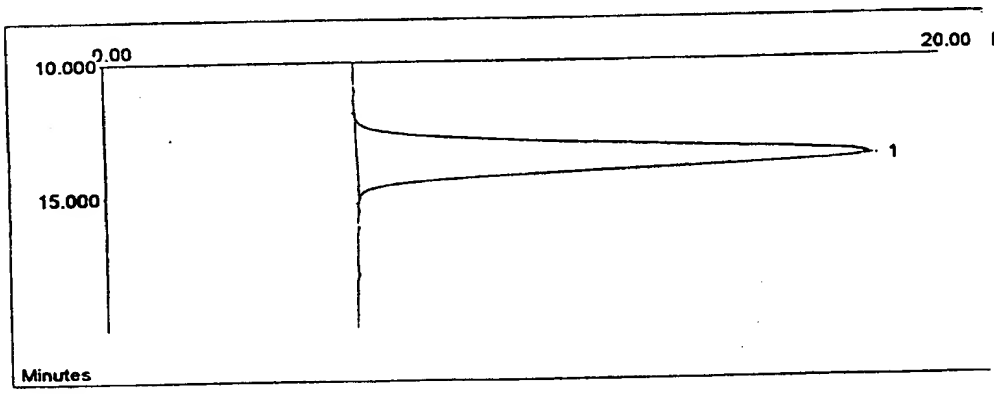
11. The use of a product according to claim 9 or a formulation according to claim 10, in the manufacture of a medicament for use in medical therapy.

12. A process according to claim 1, a product according to claim 8, a formulation according to claim 10 or a use according to claim 11, substantially as hereinbefore described with reference to the accompanying Figures and Examples.

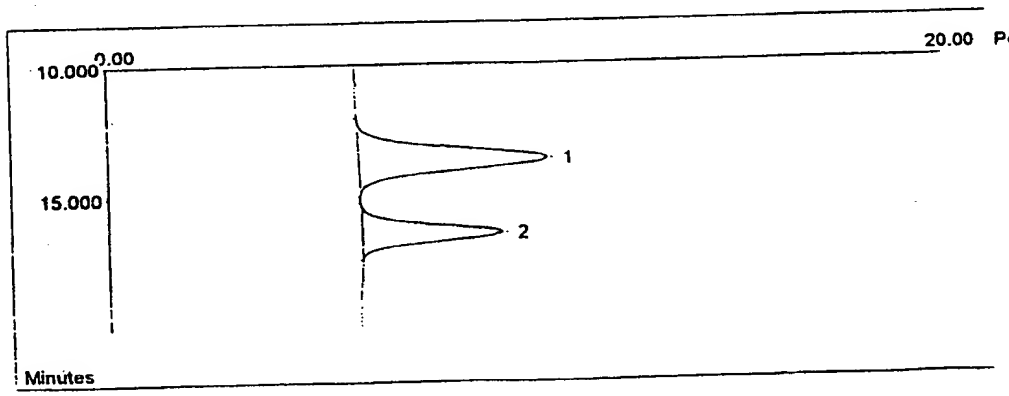
13. A reagent comprising MPEG-halide obtainable by the reaction of MPEG with tresylhalide wherein at least a part of any TMPEG produced in the reaction is removed.


10.000
0.00
15.000
20.00
Peak
RT(Mins)
1
1
16.333
Minutes

A


10.000
0.00
15.000
20.00
Peak
RT(Mins)
1
1
13.717
Minutes

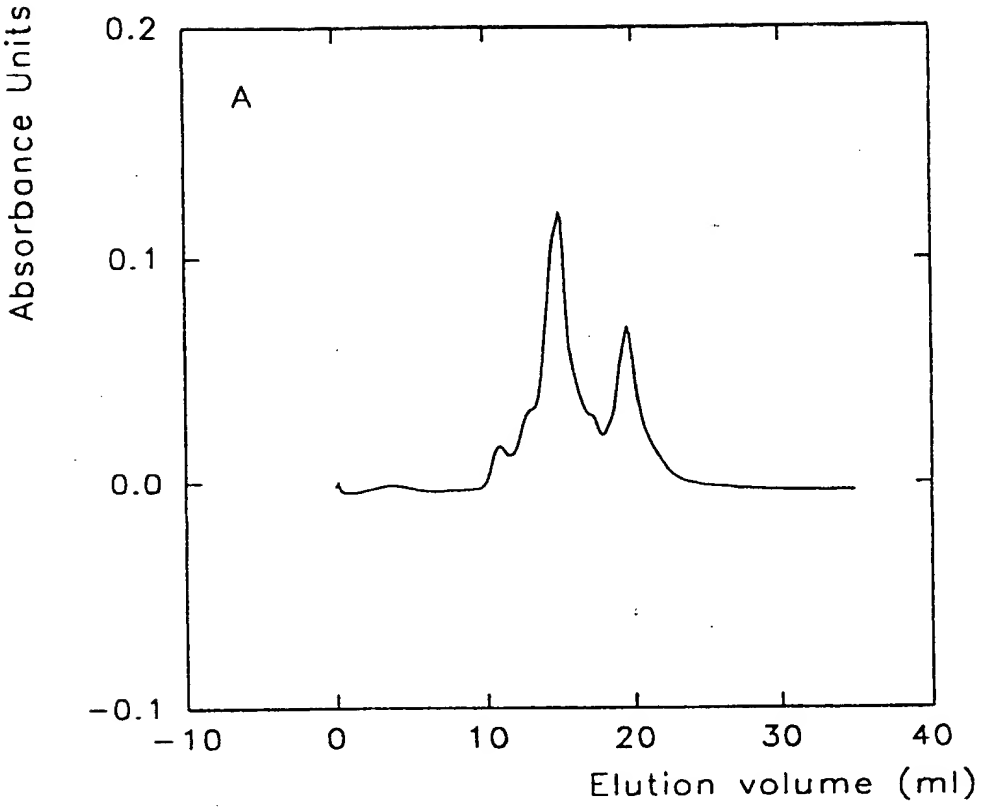
B


10.000
0.00
15.000
20.00
Peak
RT(Mins)
1
2
1
13.633
2
16.433
Minutes

C

FIGURE 1

Figure 2


Absorbance Units
0.2
0.1
0.0
−0.1
A
−10
0
10
20
30
40
Elution volume (ml)

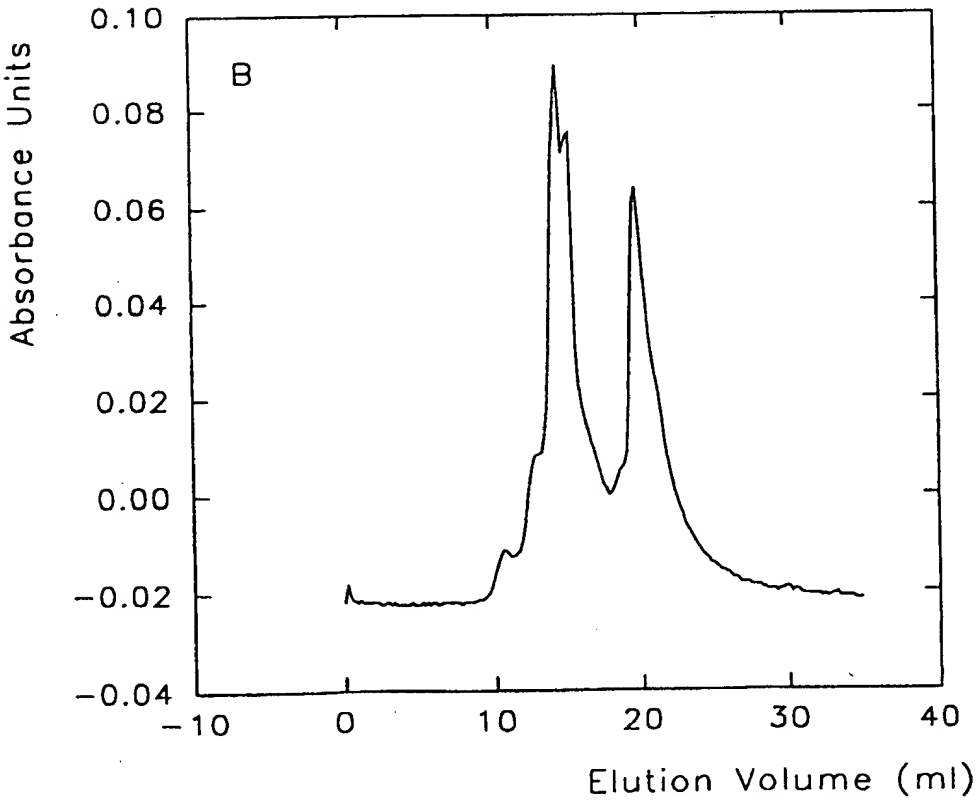

Absorbance Units
0.10
0.08
0.06
0.04
0.02
0.00
−0.02
−0.04
B
−10
0
10
20
30
40
Elution Volume (ml)

Figure 3

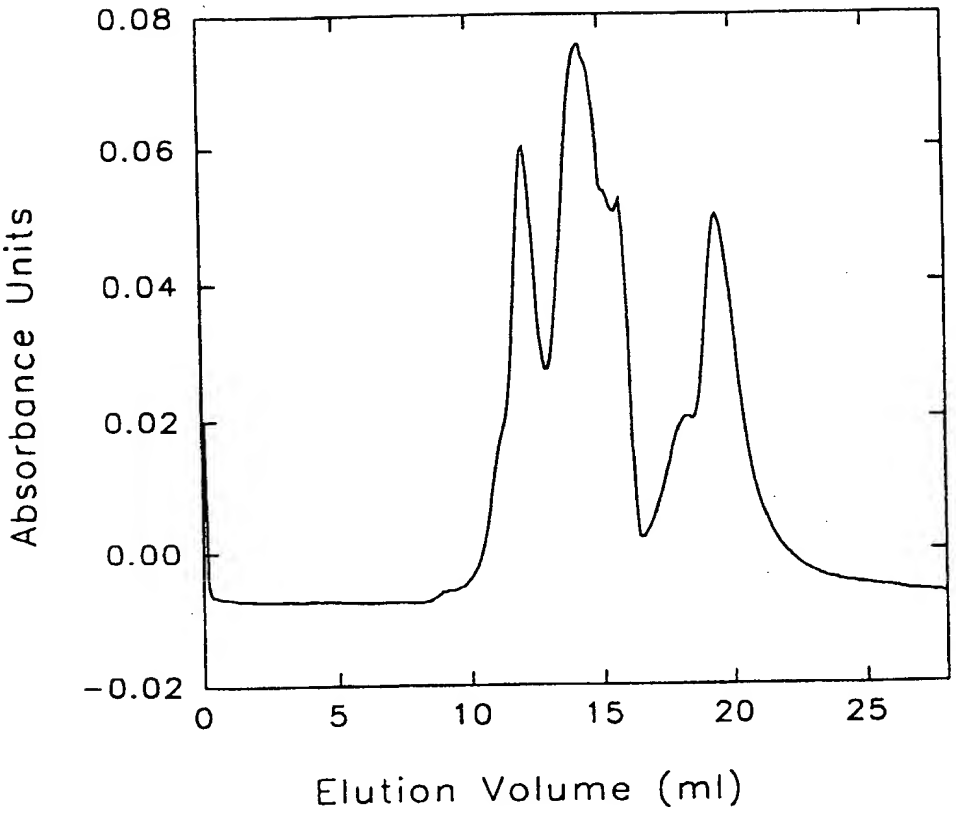

0.08
0.06
0.04
0.02
0.00
-0.02
Absorbance Units
0
5
10
15
20
25
Elution Volume (ml)

Figure 4

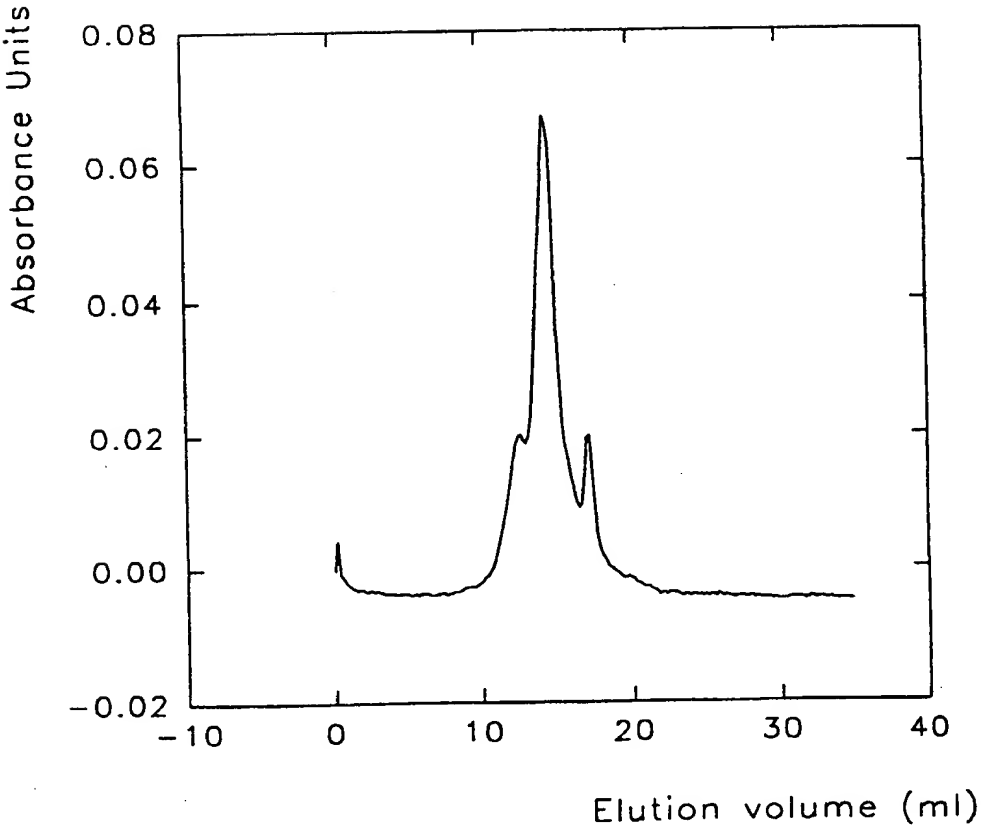

Absorbance Units
0.08
0.06
0.04
0.02
0.00
-0.02
-10
0
10
20
30
40
Elution volume (ml)

Figure 5

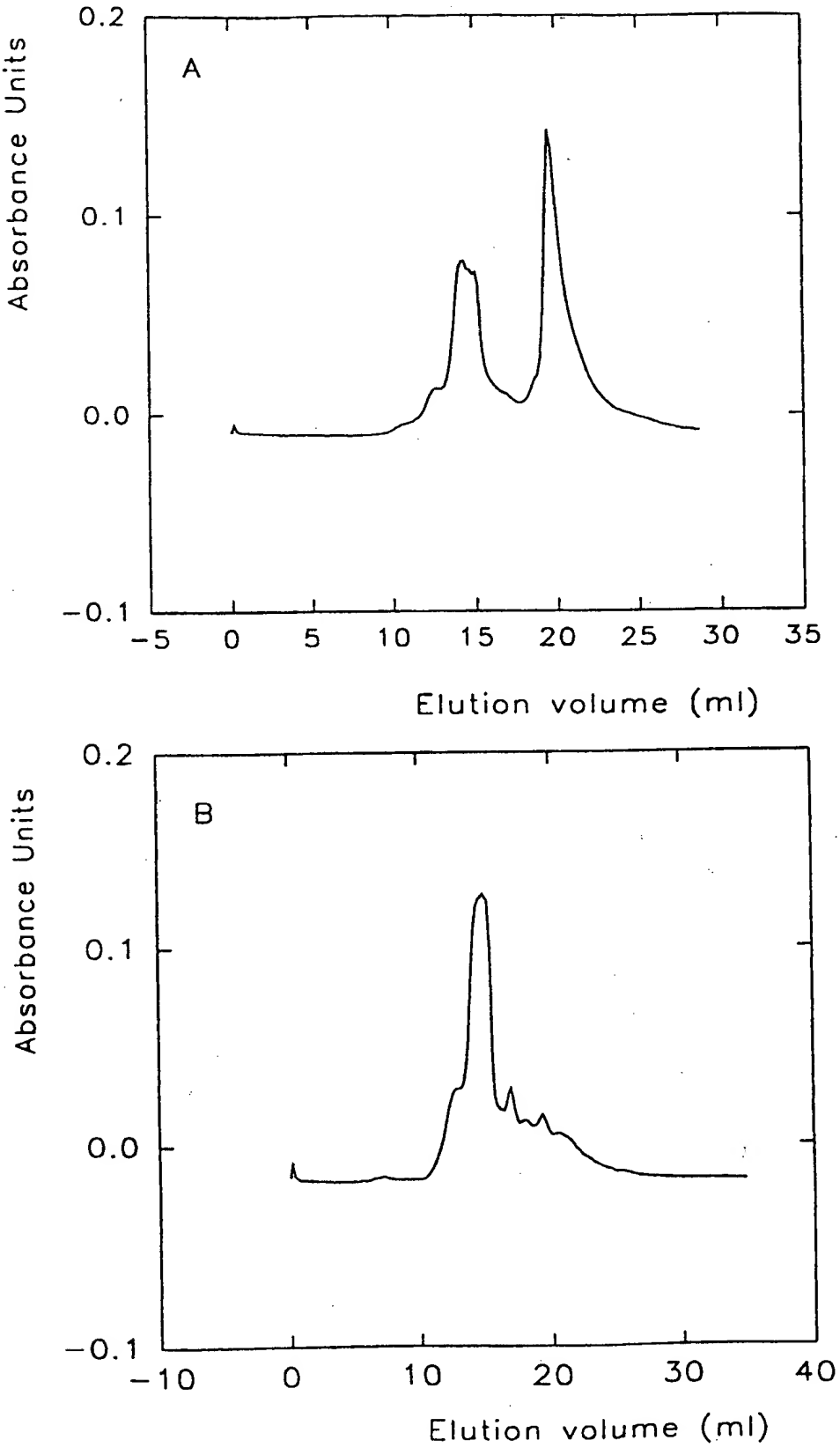

A
Absorbance Units
0.2
0.1
0.0
−0.1
−5
0
5
10
15
20
25
30
35
Elution volume (ml)
B
Absorbance Units
0.2
0.1
0.0
−0.1
−10
0
10
20
30
40
Elution volume (ml)

Figure 6

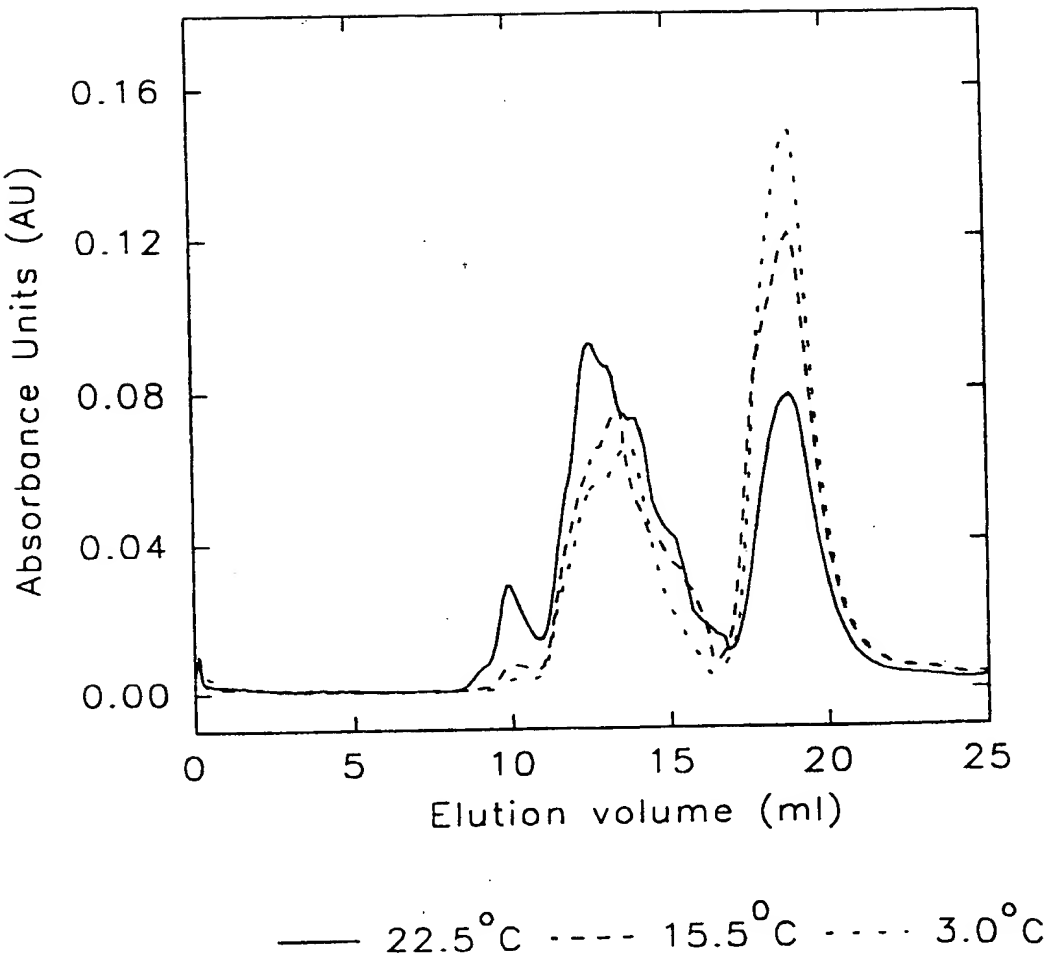

0.16
0.12
0.08
0.04
0.00
Absorbance Units (AU)
0
5
10
15
20
25
Elution volume (ml)
22.5°C
15.5°C
3.0°C

FIGURE 7

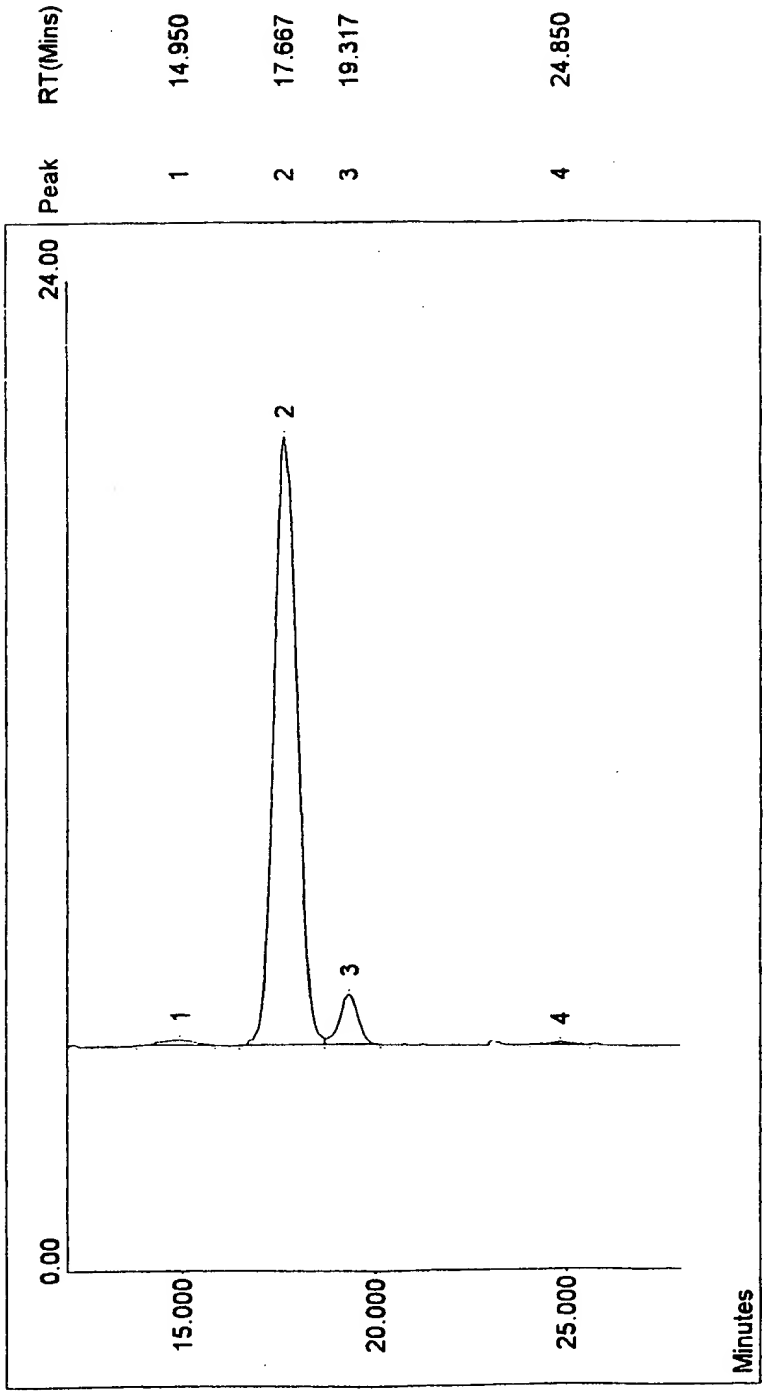

RT(Mins)
Peak
14.950
17.667
19.317
24.850
1
2
3
4
24.00
0.00
15.000
20.000
25.000
Minutes

Figure 8

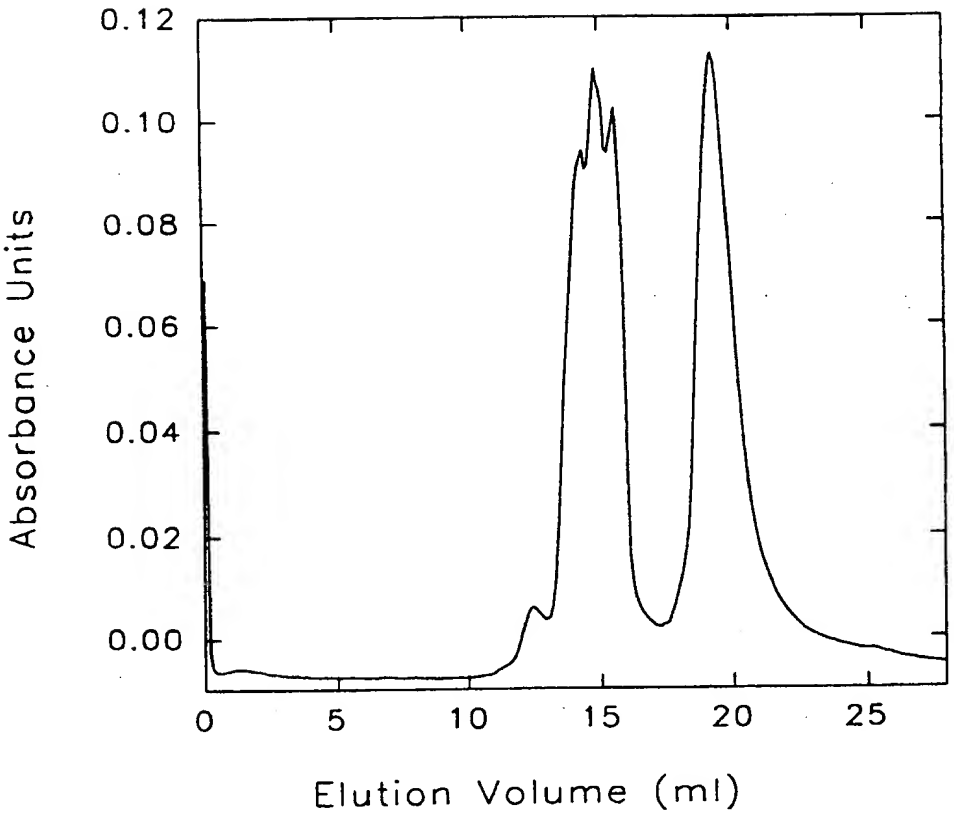

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance Units
0
5
10
15
20
25
Elution Volume (ml)

Figure 9

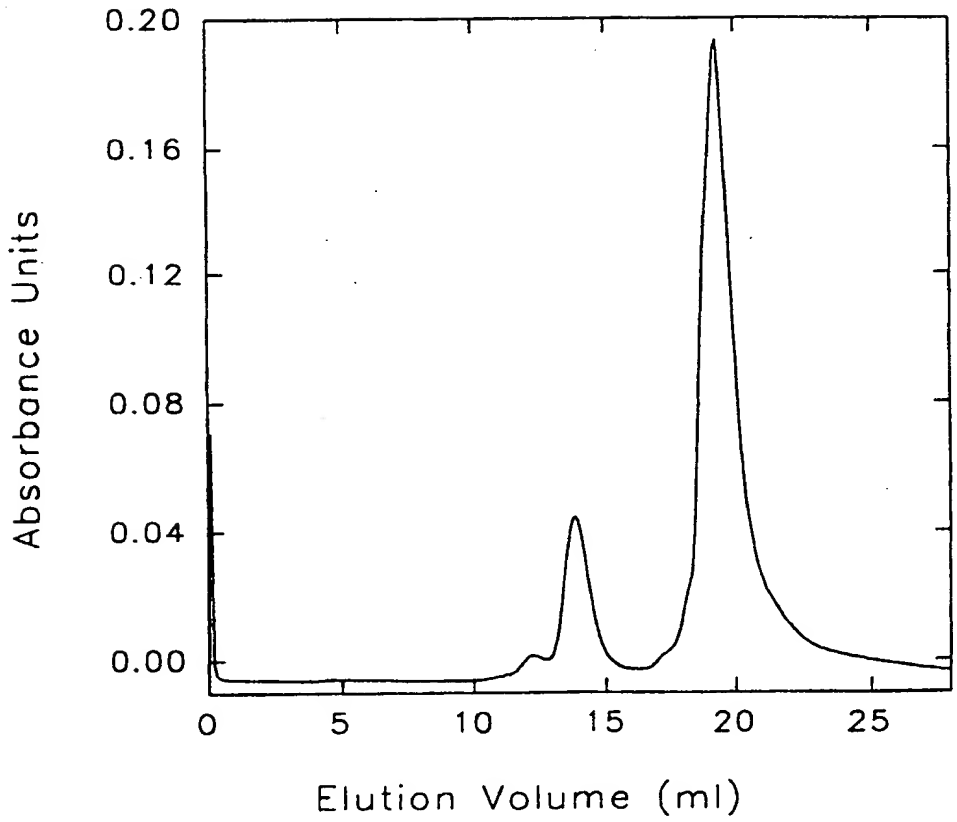

0.20
0.16
0.12
0.08
0.04
0.00
Absorbance Units
0
5
10
15
20
25
Elution Volume (ml)

Figure 10

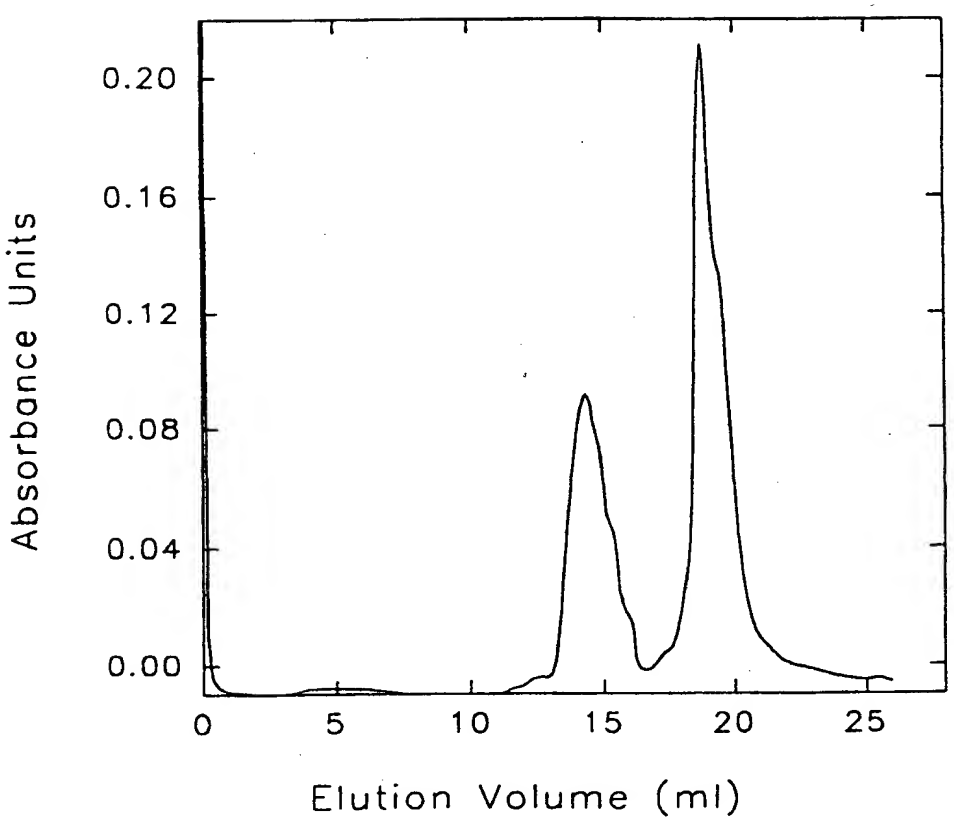

0.20
0.16
0.12
0.08
0.04
0.00
Absorbance Units
0
5
10
15
20
25
Elution Volume (ml)

FIGURE 11

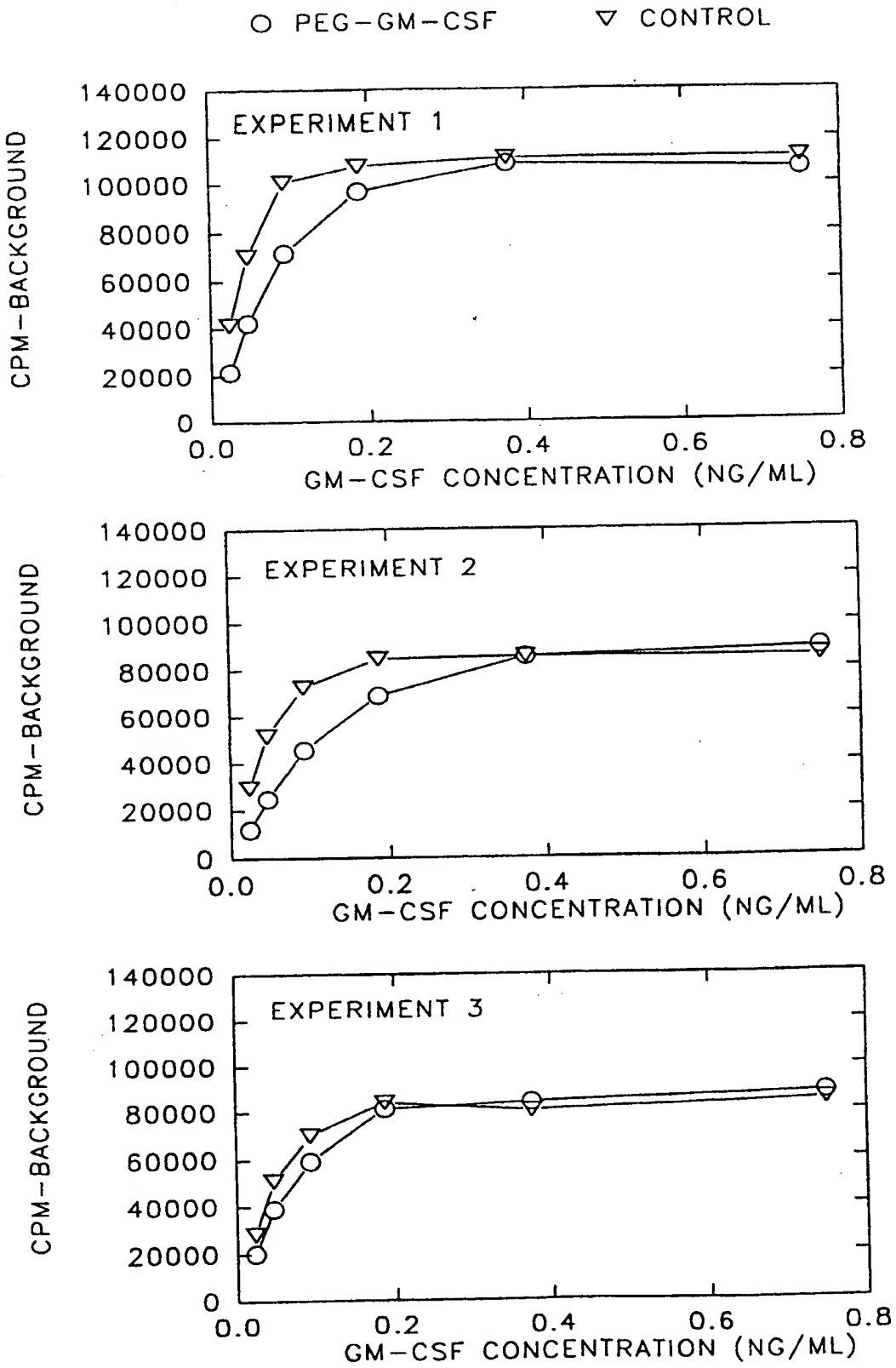

○ PEG-GM-CSF
▽ CONTROL
EXPERIMENT 1
EXPERIMENT 2
EXPERIMENT 3
CPM-BACKGROUND
GM-CSF CONCENTRATION (NG/ML)
140000
120000
100000
80000
60000
40000
20000
0
0.0
0.2
0.4
0.6
0.8

FIGURE 12

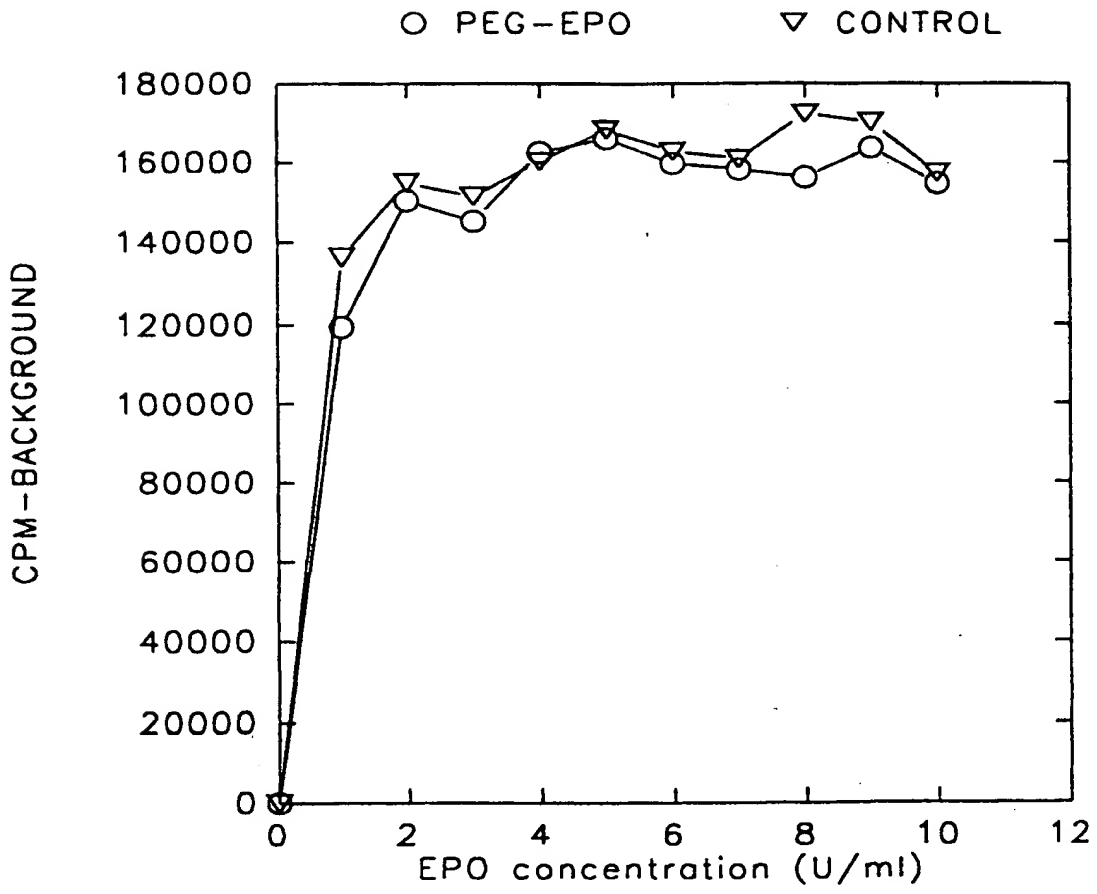

PEG-EPO
CONTROL
CPM-BACKGROUND
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
12
EPO concentration (U/ml)

FIGURE 13

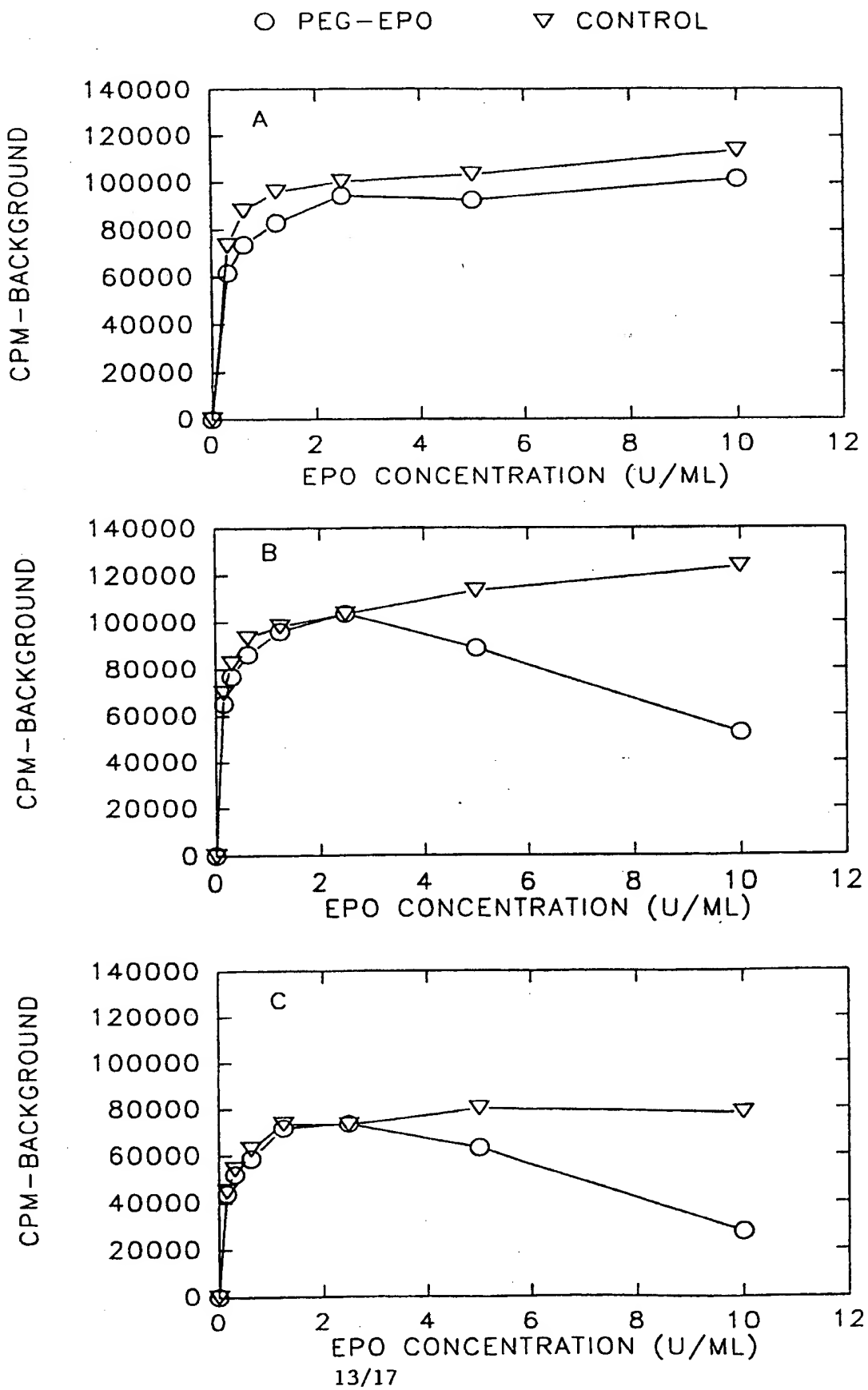

○ PEG-EPO
▽ CONTROL
A
B
C
CPM-BACKGROUND
EPO CONCENTRATION (U/ML)
0
20000
40000
60000
80000
100000
120000
140000
0
2
4
6
8
10
12

Figure 14

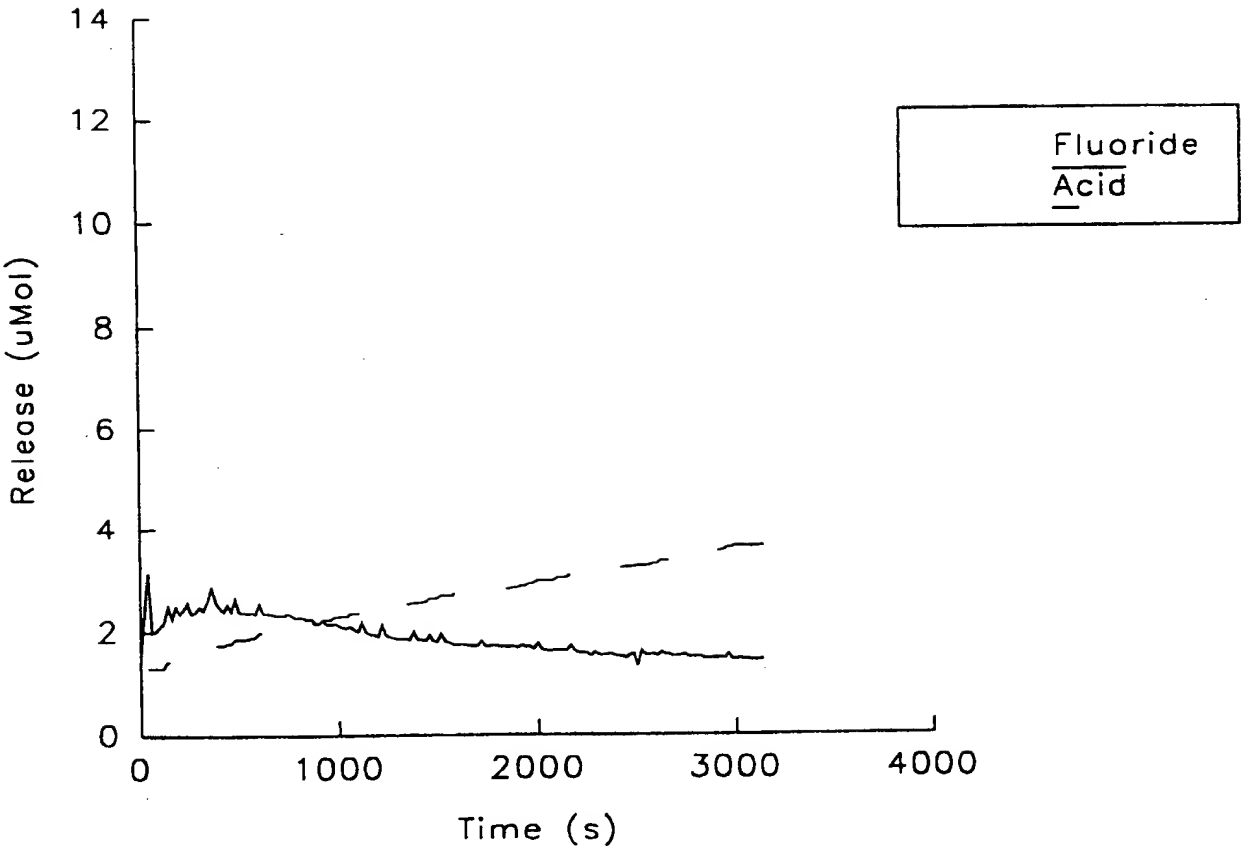

14
12
10
8
6
4
2
0
Release (uMol)
0
1000
2000
3000
4000
Time (s)
Fluoride
Acid

Figure 15

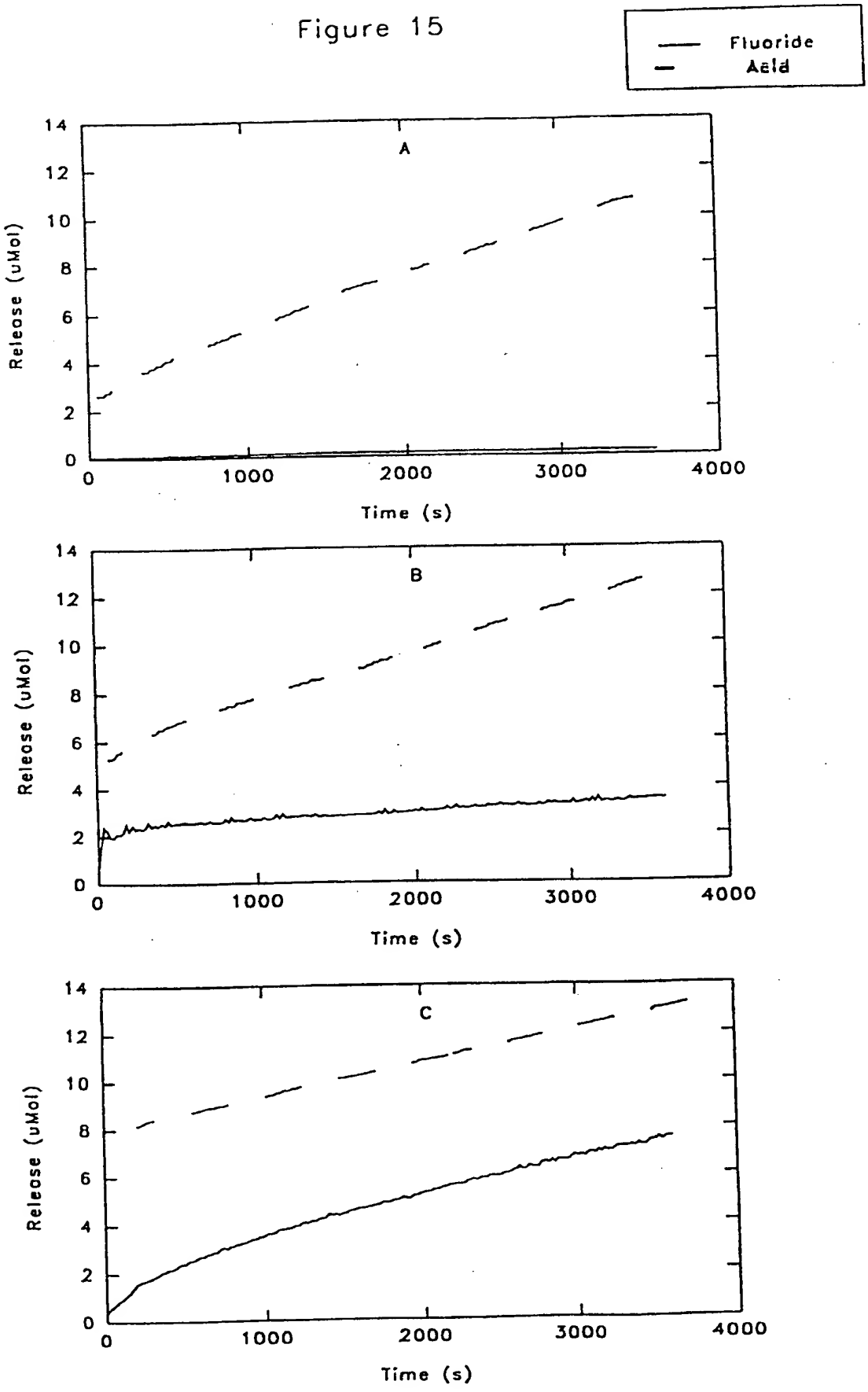

Fluoride
Acid
A
B
C
Release (uMol)
Time (s)
0
2
4
6
8
10
12
14
1000
2000
3000
4000

Figure 16

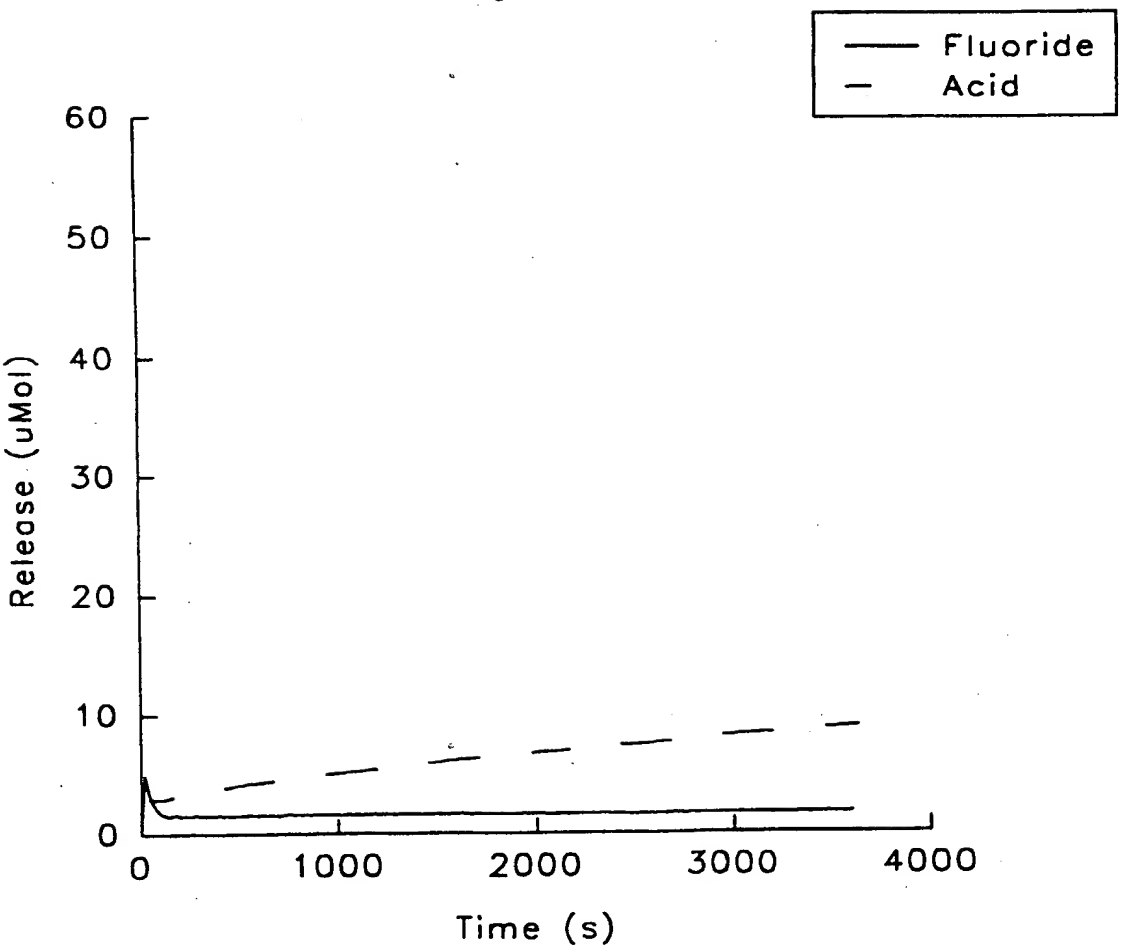
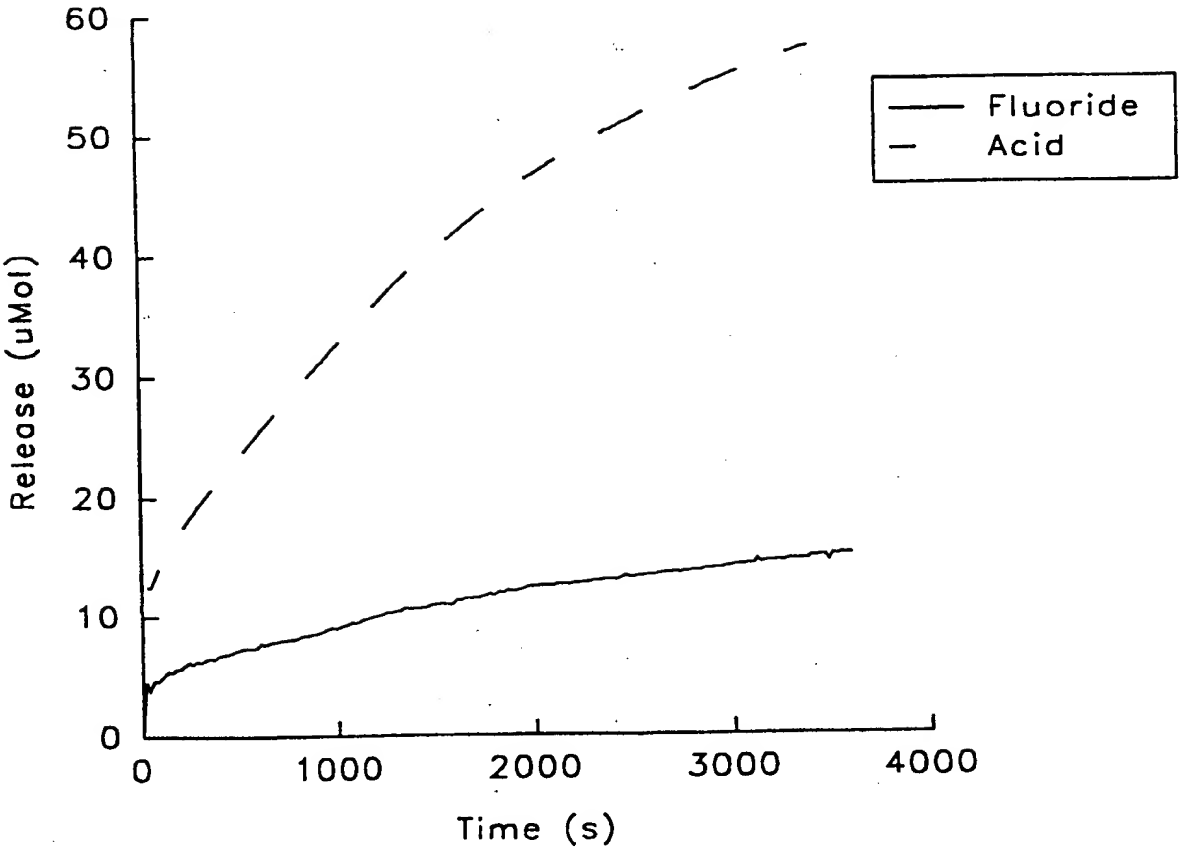

Fluoride
Acid
60
50
40
30
20
10
0
Release (uMol)
0
1000
2000
3000
4000
Time (s)

Figure 17


60
50
40
30
20
10
0
Release (uMol)
0
1000
2000
3000
4000
Time (s)
Fluoride
Acid

INTERNATIONAL SEARCH REPORT

Internatio. Application No
PCT/GB 98/00253

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 06058 A (ROYAL FREE HOSP SCHOOL MED ;FRANCIS GILLIAN ELIZABETH (GB); FISHER) 2 March 1995 see abstract; claims 8-11,15-18,22 ---	1-13
X	FRANCIS G E ET AL: "POLYETHYLENE GLYCOL MODIFICATION: RELEVANCE OF IMPROVED METHODOLOGY TO TUMOUR TARGETING" JOURNAL OF DRUG TARGETING, vol. 3, 1996, pages 321-340, XP002058312 see page 323, column 2 - page 324, column 1 see page 325; figures 1-3	1-13
A	see table 1 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 June 1998	15. 07. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gonzalez Ramon, N

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DELGADO C. ET AL: "Polymer derivatized proteins: analytical and preparative problems" PHARMACEUTICAL SCIENCES, vol. 3, 1997, pages 59-66, XP002068522 see page 60, column 2; figures 1,2,4 ---	1-13
X	WO 90 04650 A (ROYAL FREE HOSP SCHOOL MED) 3 May 1990 see page 7-8; figure 1 ---	1,2,4,5, 7-13
X	ZALIPSKY S: "CHEMISTRY OF POLYETHYLENE GLYCOL CONJUGATES WITH BIOLOGICALLY ACTIVE MOLECULES" ADVANCED DRUG DELIVERY REVIEWS, vol. 16, no. 2/03, 1995, pages 157-182, XP002037428 see page 161, column 2, paragraph 3; figure 2; tables 1,3,4 see page 176 - page 177 ---	1,2,4,5, 7-13
X	ZALIPSKY S.: "Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates" BIOCONJUGATE CHEM., vol. 6, no. 2, 1995, pages 150-165, XP002068523 cited in the application see page 151, column 1, paragraph 3; figure 1; table 2 ---	1-3,6-13
X	WO 95 34326 A (KOHNO TADAHIKO ;KACHENSKY DAVE (US); HARRIS MILTON (US)) 21 December 1995 see page 16, line 2-8 see page 19, line 1-7; example 1 ---	1-13
X	EP 0 539 167 A (ORTHO PHARMA CORP) 28 April 1993 see page 5, line 56 - page 6, line 2; examples 2,6 see page 10, line 1-25; claim 21 ---	1,2,4,5, 7-13
A	VERONESE F. M. ET AL: "A comparative study of enzymatic, structural and pharmacokinetic properties of superoxide dismutase isolated from two sources and modified by monomethoxypolyethylene glycol using different methods of coupling" ANNALS N. YORK ACAD. SCI., vol. 613, 1990, NEW YORK, pages 468-474, XP002068524 see page 469, paragraph 3; figure 1; tables 1,2 ---	1-13

-/--

INTERNATIONAL SEARCH REPORT

Internatio. Application No
PCT/GB 98/00253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GAERTNER H F ET AL: "SITE-SPECIFIC ATTACHMENT OF FUNCTIONALIZED POLY(ETHYLENE GLYCOL) TOTHE AMINO TERMINUS OF PROTEINS" BIOCONJUGATE CHEMISTRY, vol. 7, no. 1, 1996, pages 38-44, XP000646874 see abstract; figure 1 -----	1-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1,2,7-12
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 98/00253

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 1,2,7-12

In view of the large number of compounds, which are defined by the general definition in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application. (see Guidelines, Chapter III, paragraph 2.3).

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati . Application No
PCT/GB 98/00253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9506058 A	02-03-1995	EP 0714402 A	05-06-1996
		JP 9504515 T	06-05-1997
WO 9004650 A	03-05-1990	EP 0439502 A	07-08-1991
		JP 4501356 T	12-03-1992
WO 9534326 A	21-12-1995	AU 2828695 A	05-01-1996
		BG 101095 A	30-09-1997
		BR 9507999 A	12-08-1997
		CA 2191971 A	21-12-1995
		CZ 9603576 A	12-03-1997
		EP 0758906 A	26-02-1997
		FI 964985 A	16-12-1996
		NO 965342 A	14-02-1997
		PL 317894 A	28-04-1997
		SK 159596 A	06-08-1997
EP 0539167 A	28-04-1993	AU 668841 B	16-05-1996
		AU 1168495 A	01-06-1995
		AU 658231 B	06-04-1995
		AU 2718392 A	22-04-1993
		CA 2080891 A	22-04-1993
		FI 924747 A	22-04-1993
		JP 5214092 A	24-08-1993
		NO 971085 A	22-04-1993
		NZ 244778 A	25-03-1994
		ZA 9208099 A	20-04-1994

Proc. Nat. Acad. Sci.
Vol. 71, No. 3, pp. 947–951, March 1974

Photochemical Attachment of *lac* Repressor to Bromodeoxyuridine-Substituted *lac* Operator by Ultraviolet Radiation

(catabolite gene activator protein/protein–DNA interaction/nitrocellulose-filter assay)

SYR-YAUNG LIN AND ARTHUR D. RIGGS

City of Hope National Medical Center, Duarte, California 91010

Communicated by Matthew Meselson, October 27, 1973

ABSTRACT **The transducing phage λh80*dlac* carries the *lac* operator, whereas wild-type λh80 does not. We find that in high salt (0.18 M KCl), ultraviolet radiation causes the formation of a very stable complex between repressor and 5-bromodeoxyuridine (BrdU)-substituted λh80*dlac* but not to BrdU-λh80 DNA. Studies with inducers of the *lac* operon confirm the specificity of attachment. In low salt (0.01 M KCl), ultraviolet radiation will also attach repressor nonspecifically to BrdU-λh80 DNA. The stability of the complex suggests that covalent bonds are formed. We also report that another regulatory protein, the catabolite gene activator protein, can be attached similarly to DNA.**

Smith first noted that ultraviolet (UV) radiation cross-links protein to DNA, both *in vivo* and *in vitro* (1). The experimental evidence for cross-linking was that after UV treatment, DNA was not extractable from sodium dodecyl sulfate (SDS)–protein precipitates. This work has been reviewed (2). Proteins known to bind to DNA were not studied. Recently, Markovitz (3) demonstrated that UV irradiation results in covalent bond formation between DNA polymerase and DNA. Stimulated by the work of Markovitz, we tried to demonstrate the specific cross-linking of *lac* repressor to *lac* operator in λh80*dlac* DNA. These experiments were not successful until 5-bromodeoxyuridine (BrdU)-substituted λh80*dlac* DNA was used. We report here the photochemical attachment of *lac* repressor specifically to BrdU-substituted *lac* operator.

METHODS

We prepared *lac* repressor (i^{superq}) from strain M96 following the procedure of Müller-Hill, Beyreuther, and Gilbert (4). To ensure purity, additional chromatography on DEAE-Sephadex was done (5). The preparation was free of impurities detectable by SDS–acrylamide gel electrophoresis and all DNA-binding activity (including photochemical cross-linking) sedimented in a sucrose gradient as *lac* repressor. The nitrocellulose filter assay for repressor–DNA complexes has been described in detail (6, 7). Because of somewhat lower background and better reproducibility, we are now using type HAMK filters from the Millipore Corp. The basic procedures for preparing BrdU-substituted λh80*dlac* [^{32}P]DNA and λh80 [^{32}P]DNA are published (8). For this work the thymine-requiring double lysogen, JG108 ($\lambda h_{80}C_{1857}S_{68}dlac$, $\lambda h_{80}C_{1857}S_{68}$), was grown in medium containing 10 μg/ml of BrdU and 0.2 μg/ml of thymidine for 30 min prior to heat induction. This procedure leads to about 90% substitution BrdU for thymidine as estimated by buoyant density measurements in CsCl (9).

For most experiments, ultraviolet light treatment was at a distance of 11 cm from a short wavelength mineral light (Ultraviolet Products, model UVS-11). The sample (0.75 ml) was in 0.5 × 2-inch polyallomer tubes situated directly below the UV lamp. Irradiation was usually done at room temperature (25°) in buffer I, which contains: 10 mM KCl, 10 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% (v/v) dimethylsulfoxide, and 50 μg/ml of BSA. The BSA was heat treated at 70° for 2 hr at pH 9.0. UV dosage was measured with an ultraviolet meter (Ultraviolet Products model J-225). Test tubes with the bottom cut out were used to estimate the dose actually received by the sample.

RESULTS

The specific binding of *lac* repressor to *lac* operator has been firmly established (10, 11) and studied in detail using nitrocellulose filters to asay for repressor–operator complexes (cf. 12–14). Repressor binding to operator is eliminated by isopropyl-β-D-thiogalactoside (IPTG), a good inducer of the *lac* operon. IPTG at 10^{-3} M causes preformed repressor–operator complexes to dissociate in a few seconds, even in low ionic strength buffers (ref. 13, and our unpublished data). The binding of repressor to operator is also very sensitive to salt concentration and preformed repressor–operator complexes dissociate quickly in high salt (13). *Lac* repressor has a relatively weak, but nonetheless measurable, affinity for DNA not containing the *lac* operator (7). Repressor binding to nonoperator DNA is sensitive to ionic strength, but is not affected by IPTG (7).

The results above were obtained using normal, unsubstituted DNA. However, as shown in Fig. 1, the binding of repressor to BrdU-substituted DNA is basically similar. Specificity for operator is easily demonstrated because IPTG eliminates the binding (Fig. 1 and ref. 8) and no binding is observed if DNA without the *lac* operon (BrdU-λh80 [^{32}P]DNA) is used (data not shown). An important difference between normal and BrdU-substituted operator is that the rate of dissociation of *lac* repressor is ten times slower from the latter (8). Recent work (Lin and Riggs, unpublished) has established that the equilibrium affinity of *lac* repressor for both operator and nonoperator BrdU-substituted DNA is increased about one order of magnitude.

Specific attachment. Fig. 2A shows that, in 0.18 M KCl, UV irradiation leads to the formation of IPTG-resistant complexes between repressor and BrdU-λh80*dlac* DNA. The zero-

Abbreviations: BSA, bovine-serum albumin; BrdU, 5-bromodeoxyuridine; CAP, catabolite gene activator protein; IPTG, isopropyl-β-D-thiogalactoside; SDS, sodium dodecyl sulfate; UV, ultraviolet.

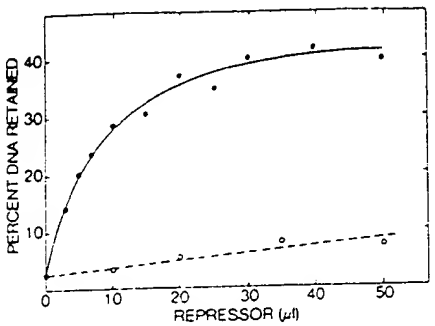


FIG. 1. Binding of *lac* repressor to BrdU-substituted λh80-*dlac* [^{32}P]DNA. Various amounts of repressor were added to 0.4 ml of buffer I, containing 25 ng of BrdU-substituted λh80-*dlac* [^{32}P]DNA. The reaction mixtures were incubated at room temperature for at least 30 min before filtration on Millipore filters. In some tubes, IPTG was added to a final concentration of 10^{-3} M before adding repressor. Each point is the average of three filters. ●——●, without IPTG. O- - -O, IPTG added to a final concentration of 1 mM before adding repressor.

time point serves as a control to establish that without UV irradiation, IPTG completely eliminates repressor–operator complexes. Repressor must be present during irradiation; prior irradiation of the DNA does not lead to stable complex formation. Under these conditions, no stable complex is formed with BrdU-λh80 DNA. The BrdU-λh80 DNA was

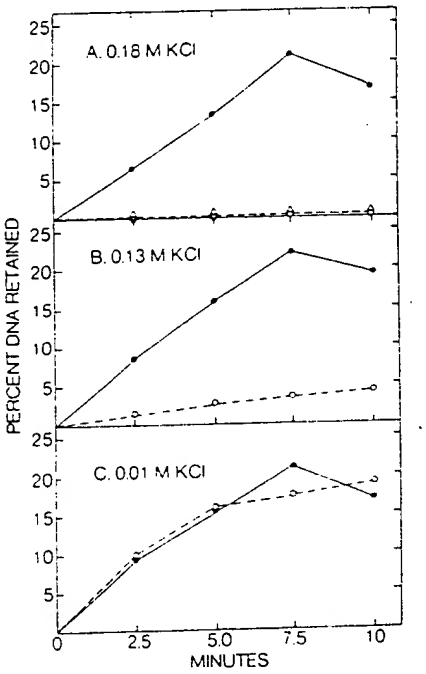


FIG. 2. Formation of IPTG-stable complexes between *lac* repressor and BrdU-DNA by UV radiation. *Lac* repressor (0.5 ng) was added to 50 ng of BrdU-DNA in 0.75 ml of buffer I adjusted to contain the indicated concentration of KCl. After 40 min to allow repressor to bind to operator, the samples were UV-irradiated at room temperature (25°) for the indicated times. IPTG was then added to a final concentration of 1 mM. After at least 10 min, 0.9 ml of buffer I containing 50 μg of sonicated salmon-sperm DNA was added, and 0.5-ml aliquots were filtered in triplicate through Millipore filters. DNA retention observed when UV irradiation preceded the addition of repressor (less than 5%) has been subtracted. (●—— ●), BrdU-λh80*dlac* [^{32}P]-DNA. (O- - -O), BrdU-λh80 [^{32}P]DNA. (Δ- - -Δ), BrdU-λh80-*dlac* [^{32}P]DNA, 1 mM IPTG present *during* UV irradiation.

TABLE 1. *Effect of various reaction conditions on cross-linking of repressor to operator*

A		B	
Reaction condition	Percent control	Reaction condition	Percent control
Minus repressor	2	10^{-4} M IPTG	12
0°	26	10^{-3} M IPTG	1
25°	100	10^{-3} M methyl-β-D-thiogalactoside	10
37°	74	10^{-2} M melibiose	8
Minus dimethyl-sulfoxide	110	10^{-2} M lactose	131
0.01 M dithio-threitol	92	10^{-4} M phenyl-β-D-thiogalactoside	90
pH 7	75	10^{-4} M o-nitro-phenyl-β-D-fucoside	106
pH 8	84	10^{-2} M glucose	112
		10^{-2} M galactose	36

A. Repressor (2 ng) was added to 200 ng of BrdU-λh80*dlac* [^{32}P]DNA in 0.3 ml of buffer I, containing 0.18 M KCl and adjusted to the various conditions indicated. UV irradiation (10 min) and IPTG treatment were done as in the legend of Fig. 2. Before filtration the reaction mixture was diluted with 3.0 ml of buffer I containing 50 μg of sonicated salmon-sperm DNA. Samples (1 ml) were filtered in triplicate.

B. The basic procedure for these experiments is described in the legend of Fig. 2. Buffer I containing 0.18 M KCl and the indicated ligand was used. For each ligand, an experiment with no repressor present during UV irradiation was done to establish that the ligand alone caused no filter retention of DNA.

prepared identically to the BrdU-λh80*dlac* DNA (the phages were from a double lysogen and separated in CsCl) and had the same degree of BrdU substitution (90%) as measured by buoyant density. Sucrose gradient centrifugation experiments established that the molecular weights of the BrdU-λh80 and BrdU-λh80*dlac* DNAs were the same. Therefore, these data provide strong evidence for specific attachment of *lac* repressor to *lac* operator. Another argument for specificity will be developed below when the action of effector ligands is considered.

Nonspecific Attachment. In low salt no evidence for specific cross-linking to operator is seen. Fig. 2*C* shows that in 0.01 M KCl, IPTG-stable complexes form equally well with BrdU-λh80 and BrdU-λh80*dlac* DNA. Without UV treatment, filter retention only of BrdU-λh80*dlac* DNA is observed. We interpret these results as follows: The *lac* repressor does have measurable general affinity for DNA and this affinity for nonoperator DNA is much higher in low salt (7). Although the affinity of repressor for nonoperator DNA is much less than for operator, the effective concentration of nonoperator-binding sites is very high. In low salt, the probability of repressor being bound to BrdU-λh80 DNA is very high (see ref. 7, and note that the affinity of repressor for BrdU-substituted DNA is about 10 times greater than for unsubstituted DNA). Apparently this weakly bound repressor is not able to cause filter retention of the DNA. After UV treatment and the formation of a more stable complex, the DNA is retained on filters.

Effector Ligands. Many effector ligands, mostly galactosides, are known to interact with the *lac* repressor and affect its affinity for operator. Some are inducers and decrease bind-

TABLE 2. *Stability of repressor–DNA complexes formed during UV irradiation*

Treatment after UV	Ratio of treated to control	
	Nonoperator (λh80 DNA)	Operator (λh80*dlac* DNA)
10^{-3} M IPTG[a]	1	1
1 M KCl[b]	0.95	0.95
80°, 30 min[c]	1.04	0.33
0.2 N NaOH[d]	0.82	—[d]
Pronase[e]	0	0

The procedure through irradiation was as described in the legend of Fig. 2. For the experiments with nonoperator [^{32}P]-DNA (BrdU-λh80), buffer I was used. For experiments where binding was to operator, BrdU-λh80*dlac* [^{32}P]DNA was used, and the buffer contained 0.18 M KCl. After UV irradiation (7.5 min), various treatments were given before filtration.

[a] At least 10 min before filtration, IPTG was added to a final concentration of 10^{-3} M. The control received no IPTG.

[b] KCl was added to a final concentration of 1 M. After at least 20 min at 25°, 3 ml of buffer I containing 50 μg of salmon-sperm DNA was added and 1-ml samples were filtered. The control was with water added in place of the KCl solution.

[c] λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, 400 μg of BSA was added (to protect from adsorption to the walls of the test tube) and the reaction mixture heated at 80° for 30 min. The control was not heated. λh80*dlac* DNA was not sonicated and only 0.5 ng of repressor was used.

[d] When nonoperator binding was studied, λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, NaOH was added to a final concentration of 0.2 N. The solution was incubated at 25° for 10 min and then neutralized with HCl. For the control, NaCl was added instead of NaOH. When operator binding was studied, the λh80*dlac* DNA could not be sonicated and such high backgrounds (no repressor) were obtained after denaturation as to render the experiment meaningless. Experiments where the DNA was renatured overnight at 65° were also unsuccessful because of high backgrounds.

[e] Pronase (20 μg) was added and the reaction mixture incubated at 37° for 10 min. The control got no Pronase, but was incubated at 37° for 10 min.

ing affinity; others increase affinity and are known as anti-inducers (12). IPTG, an inducer, is known to cause slight conformational changes in the *lac* repressor (15–17). Under conditions where specific binding to BrdU-λh80*dlac* DNA is seen, the presence of IPTG during irradiation completely eliminates the formation of stable complex (Fig. 2 and Table 1). It was conceivable that IPTG was acting, not by causing a conformational change in the repressor, but rather in some nonspecific way, perhaps by reacting with the free radicals produced during UV irradiation. Therefore, we checked the effect of other galactosides. As illustrated in Table 1, only ligands known to inhibit repressor binding to operator are effective. Anti-inducers such as glucose, *o*-nitrophenylfucoside, and phenylthiogalactoside (12) do not inhibit stable complex formation. Lactose, a disaccharide which recently has been shown to be an anti-inducer (18), does not inhibit, whereas melibiose, a disaccharide that acts as an inducer (12), greatly reduces the formation of stable complexes. These results establish that the effect of these ligands is mediated through the *lac* repressor.

In contrast, Fig. 3 shows that in low salt, where nonspecific interactions predominate, the presence of IPTG before and

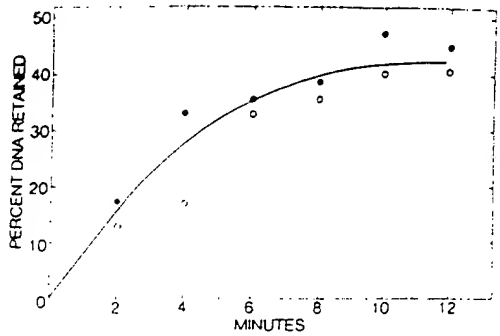


FIG. 3. The effect of IPTG on the photochemical attachment of *lac* repressor to wild-type BrdU-λh80 DNA. *Lac* repressor (1.0 ng) was added to 50 ng of BrdU-λh80 [^{32}P]DNA in 0.75 ml of buffer I. In one set of reaction mixtures, 1 mM IPTG was present. After 20 min at room temperature, the reaction mixtures were UV irradiated for the indicated times. To those samples with no ligand present during UV irradiation, IPTG was then added to a concentration of 1 mM. After an additional 10 min, 0.2-ml samples were filtered, in triplicate, through Millipore filters. (●——●) 1 mM IPTG present before and during UV treatment. (O- - -O) 1 mM IPTG added after UV treatment.

during irradiation does not affect the formation of stable complexes. This result is in keeping with our earlier observations that IPTG does not affect the binding of repressor to nonoperator DNA (7). Apparently, the conformational changes induced in the repressor by galactosides only affect binding to operator. Since in 0.18 M KCl, IPTG does eliminate stable complex formation, it follows that, under these conditions, attachment is to the *lac* operator.

Chemistry and Efficiency of Attachment. Markovitz found that UV irradiation induces the formation of DNA polymerase–DNA complexes that are resistant to high salt, phenol, heat, and 0.1 M NaOH (3); he concluded that a covalent bond between DNA and protein had been established. Photoinduced protein–DNA complexes also are known to be resistant to SDS (2, 25). Under conditions where repressor is binding nonspecifically to BrdU-substituted DNA, we have obtained similar results (Table 2). The complexes also are stable to SDS (Table 3). These results strongly suggest that UV treatment covalently attaches repressor to DNA.

There is no reason to think that the mechanism of photochemical attachment of repressor to operator DNA is fundamentally different from attachment to nonoperator DNA. However, experiments to establish this point have proven more difficult, because under conditions where specific attachment of repressor to operator occurs, only one repressor protein is bound for each BrdU-λh80*dlac* DNA molecule (30×10^6 molecular weight). The filter assay requires that this protein cause the DNA to be retained on the nitrocellulose filters. Experiments where strand separation occurs (0.2 M NaOH and boiling temperatures) have not been successful. UV irradiation is known to introduce single strand breaks in BrdU-DNA, so after strand separation, the majority of DNA fragments would not be expected to have repressor peptides attached. Treatment at 80° causes a drop in DNA filter retention to a value about one-third that of the control. A denatured subunit attached to DNA may not be as effective in causing the DNA to be retained on the filters as the native tetramer. Although the above experiments were ambiguous, others were more definitive. The data in Table 3 provide

TABLE 3. *SDS and salt stability of photoinduced repressor-DNA complexes*

Reaction condition	DNA	Percent DNA in nonaqueous phase	
		No UV	+ UV
Nonspecific (0.01 M KCl)	λh80	0.8	62
Specific (0.18 M KCl)	λh80	0.3	1.4
	λh80*dlac*	0.5	8.2

For the experiment under nonspecific conditions, 200 ng of repressor was mixed with 400 ng of BrdU-λh80 [^{32}P]DNA in 1.5 ml of buffer I without dimethylsulfoxide. After 10 min of UV treatment, a modification of the procedure of Smets and Cornelis (25) was applied. BSA, SDS, and NaCl were added to a final concentration of 70 μg/ml, 0.2%, and 1 M, respectively; then an equal volume of $CHCl_3$:isoamyl alcohol (12:1) was added and the mixture gently shaken at 25° for 10 min. The water phase and $CHCl_3$ phase were separated and counted. The interphase was collected by filtration through Whatman GF/C glass filters. Before counting, the filters were washed with 1 N HCl containing 0.05 M sodium pyrophosphate, and then with ethanol. For the experiments under specific conditions, the same procedure was followed, except that only 8 ng of repressor was used and buffer I contained 0.18 M KCl.

strong evidence for SDS-stable cross-linking of repressor to operator. UV irradiation in the presence of repressor increases more than 10-fold the amount of DNA trapped in $CHCl_3$ and the protein–SDS interphase. Most important is the complete resistance of the photochemical complexes to high salt and inducer concentrations (agents that would quickly and completely eliminate normal repressor–operator complexes). Therefore, we think that photochemical attachment to operator occurs as it does to nonoperator DNA with the formation of extremely stable and probably covalent bonds.

Before UV irraidation, repressor causes a maximum of about 40% of BrdU-λh80*dlac* DNA to be retained on filters (Fig. 1). Fig. 2 shows that after UV irradiation and the formation of IPTG-resistant complexes, a maximum of about 20% of the DNA is retained. Therefore, from the data shown, it is apparent that the efficiency of complex formation is at least 50%. After 7.5 min. of UV treatment, we estimate that the sample has received a dose of 8×10^3 ergs/mm^2. Markovitz found that about 50×10^3 ergs/mm^2 was necessary to covalently attach DNA polymerase to poly(dA-dT) or normal *Escherichia coli* DNA (3). The difference in energy is in keeping with the increased sensitivity of BrdU-DNA to UV irradiation (2, 20).

As illustrated by the data shown in Table 1, the photochemical attachment of repressor to operator is much less at 0° than at 25°. This result has been obtained reproducibly and can only be partially compensated for by longer irradiation times. At 37°, background (no repressor) becomes higher and more variable. To obtain reproducible results, BSA must be present, presumably to protect repressor. However, the BSA must be heat treated (see below). Dimethylsulfoxide is not essential and can be eliminated. High concentrations of dithiothreitol do not interfere with photochemical attachment to operator.

Lac repressor binds strongly to poly(dA-dT) (7), and even more tightly to poly(dA-BrdU) (21). However, we found that

TABLE 4. *Photochemical attachment of the catabolite gene activator protein to BrdU-DNA*

Ligand	Exp. no.	Percent DNA retained: No UV, Treatment before filtering: None	No UV: KCl (1 M)	+ UV: None	+ UV: KCl (1 M)	+ UV: cGMP (3 mM)
None	1	10	0	64	60	—
	2	19	0	42	41	39
cAMP 0.3 mM	1	71	1	53	38	—
	2	45	1	44	26	29
cGMP 0.3 mM	1	—	—	—	—	—
	2	6	0	7	4	3

The reaction mixture contained the indicated ligand and 0.1 μg of BrdU-λh80*dlac* [^{32}P]DNA in 0.7 ml of buffer I. Enough CAP was added to cause 90% of the DNA to be retained on filters under our standard (no UV) condition (23). UV irradiation was for 10 min at 25° at a distance of 11 cm. After UV treatment, either KCl (1 M) or 3′:5′-cyclic GMP (3 mM) was added and the mixture was incubated at 25° for 20 min. Sonicated salmon-sperm DNA (50 μg) was added and then 2.5 ml of buffer I was added, and 1-ml samples were filtered in triplicate. In the case of no treatment before filtering, KCl or cGMP was omitted. Controls without CAP were done and subtracted as background. Neither cyclic AMP or cyclic GMP affected the background.

the photochemical attachment of repressor to poly(dA-BrdU) required higher UV doses than attachment to operator or to sonicated BrdU-λh80 DNA. A UV dose of about 50×10^3 ergs/mm^2 was required for 50% retention of [^{3}H]poly(dA-BrdU)–repressor complex on filters. Smith has observed that the rate of photochemical addition of [^{35}S]cysteine to poly(dA-dT) is considerably less than to calf-thymus DNA (19).

Photochemical attachment of repressor to normal λh80*dlac* DNA has not been successful. Initial experiments were done with intact λh80*dlac* DNA (30×10^6 daltons). Later we prepared DNA fragments of about 1×10^6 daltons, each of which contained the *lac* operator (22). With these operator enriched fragments, even 60×10^3 ergs/mm^2 gave no IPTG-resistant complexes.

Other Proteins. In this laboratory, we have studied the DNA binding properties of another regulatory protein, the catabolite gene activator protein (CAP, CR, or CGA protein) (23). The DNA-binding activity of CAP is stimulated by cAMP, but is eliminated by cGMP or by high salt (Table 4 and refs. 23 and 24). Binding specificity has not been demonstrated (23, 24). As shown in Table 4, UV treatment leads to the formation of a CAP–DNA complex that is stable to high salt and cGMP.

We also have confirmed that UV treatment will cause DNA polymerase and RNA polymerase to form salt resistant complexes with BrdU-DNA. All four DNA-binding proteins are effective in causing DNA filter retention at concentrations of 0.01 μg/ml or less (with or without covalent attachment). Other non-DNA-binding proteins (trypsin, ovalbumin, and BSA) will cause filter retention after UV treatment, but at least one 100-fold higher concentrations are needed (1 μg/ml or more). At 50 μg/ml, even BSA will cause filter retention unless it is heat treated (see *Methods*).

DISCUSSION

Markovitz (3) has shown that UV radiation covalently attaches DNA polymerase to poly(dA-dT) and to normal *E. coli* DNA. UV irradiation of BrdU-substituted DNA leads to debromination and the consequent production of highly reactive uracilyl radicals (2, 20). Therefore, it is reasonable to think that protein bound to DNA would be more readily attached to BrdU-DNA than to normal DNA. Smith (2) and Smets and Cornelis (25) have shown, in fact, that UV treatment of cells with BrdU-substituted DNA decreases the amount of DNA that can be extracted. They interpreted this as due to the formation of protein-DNA cross-links. After this paper was in preparation, we learned that Weintraub (26) has also recently obtained evidence for the cross-linking of proteins to BrdU-substituted DNA. He has found that a variety of proteins, including histones and RNA polymerase, can be attached to BrdU-substituted DNA from mammalian cells. If specific attachment occurs, involving the correct sites on the DNA and on the protein, then UV-induced cross-linking promises to be a very useful tool. However, in none of these studies was any evidence obtained for specific attachment.

We report here that the *lac* repressor can be photochemically attached to BrdU-substituted DNA and that in high salt (0.18 M KCl) the reaction is specific for *lac* operator, i.e., attachment occurs to BrdU-λh80*dlac* DNA but not to BrdU-λh80 DNA. Inducers of the *lac* operon (i.e., IPTG) prevent photochemical attachment of repressor to operator if they are present during UV irradiation, but not if added later. In low salt (0.01 M KCl), specificity is not observed; cross-linking occurs to BrdU-λh80 DNA as well as to BrdU-λh80-*dlac*, and IPTG has no effect. By analogy to previous work and from the stability of the repressor-DNA photoproduct we think that covalent bonds are formed.

We find BrdU-substitution to be essential; no attachment to normal λh80*dlac* is obtained even with much higher UV doses. There are large differences between normal and BrdU-substituted DNA in the types and numbers of photoproducts (20). These differences may be critical for the attachment of of sequence specific proteins. BrdU-substituted DNA may be advantageous, not only because of its greater photochemical reactivity, but also because the *lac* repressor binds tighter to BrdU-substituted DNA (8).

Cysteine adds photochemically to DNA (19) and Smith has shown that cysteine reacts with uracil to form 5-*S*-cysteine-6-hydrouracil (27) or with thymine to form 5-*S*-cysteine-6-hydrothymine (28). Eleven other amino acids photochemically add to uracil with cysteine, phenylalanine, tyrosine, histidine, lysine, and arginine being the most reactive (28). Polylysine also has been cross-linked by UV radiation to DNA (26). Since many amino acids can react, it seems likely that most DNA-binding proteins will be cross-linked by UV irradiation. We find that another DNA-binding regulatory protein, the catabolite gene activator protein (23, 24), also can be attached stably to DNA. We also have confirmed (26) that RNA polymerase can be cross-linked to BrdU-DNA.

Specific covalent attachment of DNA-binding proteins to their DNA substrate is obviously of great potential usefulness for identification of the DNA-binding sites of proteins and possibly in the isolation of the DNA region covered by the proteins. For mammalian cells especially, it may be useful to attach covalently chromosomal proteins to their specific sites by UV irradiation prior to disruption of the cell or nucleus. Perhaps less obvious is that covalent attachment may be useful for the *demonstration* of specific binding, which often is a major problem with DNA-binding proteins. For many proteins, binding specificity may be greatest in high salt. However, in high salt, DNA–protein complexes are detectable in sucrose gradients only if impracticably high concentrations of reactants are used; and the same is true for retention on nitrocellulose filters. By the methods described here, it is now possible to fix permanently the protein to DNA under conditions of maximum specificity.

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Photochemical Attachment of *lac* Repressor to Bromodeoxyuridine-Substituted *lac* Operator by Ultraviolet Radiation

(catabolite gene activator protein/protein-DNA interaction/nitrocellulose-filter assay)

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ABSTRACT **The transducing phage λh80*dlac* carries the *lac* operator, whereas wild-type λh80 does not. We find that in high salt (0.18 M KCl), ultraviolet radiation causes the formation of a very stable complex between repressor and 5-bromodeoxyuridine (BrdU)-substituted λh80*dlac* but not to BrdU-λh80 DNA. Studies with inducers of the *lac* operon confirm the specificity of attachment. In low salt (0.01 M KCl), ultraviolet radiation will also attach repressor nonspecifically to BrdU-λh80 DNA. The stability of the complex suggests that covalent bonds are formed. We also report that another regulatory protein, the catabolite gene activator protein, can be attached similarly to DNA.**

Smith first noted that ultraviolet (UV) radiation cross-links protein to DNA, both *in vivo* and *in vitro* (1). The experimental evidence for cross-linking was that after UV treatment, DNA was not extractable from sodium dodecyl sulfate (SDS)–protein precipitates. This work has been reviewed (2). Proteins known to bind to DNA were not studied. Recently, Markovitz (3) demonstrated that UV irradiation results in covalent bond formation between DNA polymerase and DNA. Stimulated by the work of Markovitz, we tried to demonstrate the specific cross-linking of *lac* repressor to *lac* operator in λh80*dlac* DNA. These experiments were not successful until 5-bromodeoxyuridine (BrdU)-substituted λh80*dlac* DNA was used. We report here the photochemical attachment of *lac* repressor specifically to BrdU-substituted *lac* operator.

METHODS

We prepared *lac* repressor (i^{superq}) from strain M96 following the procedure of Müller-Hill, Beyreuther, and Gilbert (4). To ensure purity, additional chromatography on DEAE-Sephadex was done (5). The preparation was free of impurities detectable by SDS–acrylamide gel electrophoresis and all DNA-binding activity (including photochemical cross-linking) sedimented in a sucrose gradient as *lac* repressor. The nitrocellulose filter assay for repressor–DNA complexes has been described in detail (6, 7). Because of somewhat lower background and better reproducibility, we are now using type HAMK filters from the Millipore Corp. The basic procedures for preparing BrdU-substituted λh80*dlac* [^{32}P]DNA and λh80 [^{32}P]DNA are published (8). For this work the thymine-requiring double lysogen, JG108 ($\lambda h_{80}C_{1857}S_{168}dlac$, $\lambda h_{80}C_{1857}S_{168}$), was grown in medium containing 10 μg/ml of BrdU and 0.2 μg/ml of thymidine for 30 min prior to heat induction. This procedure leads to about 90% substitution BrdU for thymidine as estimated by buoyant density measurements in CsCl (9).

For most experiments, ultraviolet light treatment was at distance of 11 cm from a short wavelength mineral lig (Ultraviolet Products, model UVS-11). The sample (0.75 was in 0.5 × 2-inch polyallomer tubes situated directly bel the UV lamp. Irradiation was usually done at room temper ture (25°) in buffer I, which contains: 10 mM KCl, 10 m Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreit 5% (v/v) dimethylsulfoxide, and 50 μg/ml of BSA. The BS was heat treated at 70° for 2 hr at pH 9.0. UV dosage w measured with an ultraviolet meter (Ultraviolet Produc model J-225). Test tubes with the bottom cut out were us to estimate the dose actually received by the sample.

RESULTS

The specific binding of *lac* repressor to *lac* operator has be firmly established (10, 11) and studied in detail using nitr cellulose filters to asay for repressor-operator complex (c 12–14). Repressor binding to operator is eliminated by iso propyl-β-D-thiogalactoside (IPTG), a good inducer of the *la* operon. IPTG at 10^{-3} M causes preformed repressor–operato complexes to dissociate in a few seconds, even in low ioni strength buffers (ref. 13, and our unpublished data). The bind ing of repressor to operator is also very sensitive to salt con centration and preformed repressor–operator complexes disso ciate quickly in high salt (13). *Lac* repressor has a relatively weak, but nonetheless measurable, affinity for DNA not con taining the *lac* operator (7). Repressor binding to nonoperato DNA is sensitive to ionic strength, but is not affected by IPTG (7).

The results above were obtained using normal, unsubstituted DNA. However, as shown in Fig. 1, the binding o repressor to BrdU-substituted DNA is basically similar. Specificity for operator is easily demonstrated because IPTG eliminates the binding (Fig. 1 and ref. 8) and no binding is observed if DNA without the *lac* operon (BrdU-λh80 [^{32}P]-DNA) is used (data not shown). An important difference between normal and BrdU-substituted operator is that the rate of dissociation of *lac* repressor is ten times slower from the latter (8). Recent work (Lin and Riggs, unpublished) has established that the equilibrium affinity of *lac* repressor for both operator and nonoperator BrdU-substituted DNA is increased about one order of magnitude.

Specific attachment. Fig. 2A shows that, in 0.18 M KCl, UV irradiation leads to the formation of IPTG-resistant complexes between repressor and BrdU-λh80*dlac* DNA. The zero

Abbreviations: BSA, bovine-serum albumin; BrdU, 5-bromodeoxyuridine; CAP, catabolite gene activator protein; IPTG, isopropyl-β-D-thiogalactoside; SDS, sodium dodecyl sulfate; UV, ultraviolet.

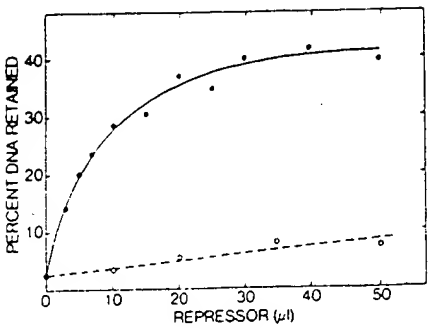

FIG. 1. Binding of *lac* repressor to BrdU-substituted λh80-*dlac* [^{32}P]DNA. Various amounts of repressor were added to 0.4 ml of buffer I, containing 25 ng of BrdU-substituted λh80-*dlac* [^{32}P]DNA. The reaction mixtures were incubated at room temperature for at least 30 min before filtration on Millipore filters. In some tubes, IPTG was added to a final concentration of 10^{-3} M before adding repressor. Each point is the average of three filters. ●——●, without IPTG. O- - -O, IPTG added to a final concentration of 1 mM before adding repressor.

time point serves as a control to establish that without UV irradiation, IPTG completely eliminates repressor–operator complexes. Repressor must be present during irradiation; prior irradiation of the DNA does not lead to stable complex formation. Under these conditions, no stable complex is formed with BrdU-λh80 DNA. The BrdU-λh80 DNA was

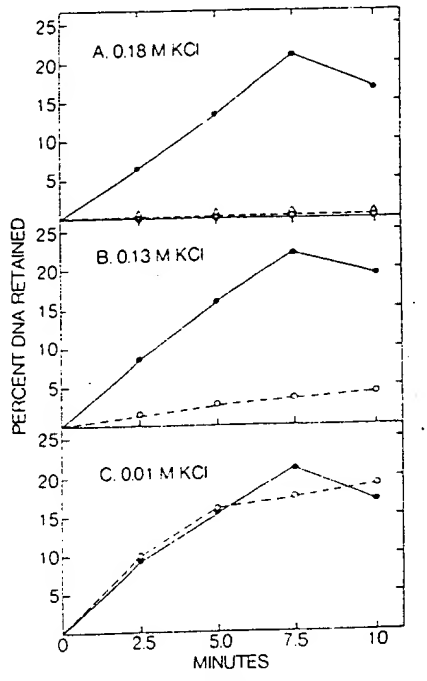

FIG. 2. Formation of IPTG-stable complexes between *lac* repressor and BrdU-DNA by UV radiation. *Lac* repressor (0.5 ng) was added to 50 ng of BrdU-DNA in 0.75 ml of buffer I adjusted to contain the indicated concentration of KCl. After 40 min to allow repressor to bind to operator, the samples were UV-irradiated at room temperature (25°) for the indicated times. IPTG was then added to a final concentration of 1 mM. After at least 10 min, 0.9 ml of buffer I containing 50 μg of sonicated salmon-sperm DNA was added, and 0.5-ml aliquots were filtered in triplicate through Millipore filters. DNA retention observed when UV irradiation preceded the addition of repressor (less than 5%) has been subtracted. (●——●), BrdU-λh80*dlac* [^{32}P]-DNA. (O- - -O), BrdU-λh80 [^{32}P]DNA. (Δ- - -Δ), BrdU-λh80-*dlac* [^{32}P]DNA, 1 mM IPTG present *during* UV irradiation.

TABLE 1. *Effect of various reaction conditions on cross-linking of repressor to operator*

A		B	
Reaction condition	Percent control	Reaction condition	Percent control
Minus repressor	2	10^{-1} M IPTG	12
0°	26	10^{-3} M IPTG	1
25°	100	10^{-1} M methyl-β-D-thiogalactoside	10
37°	74	10^{-2} M melibiose	8
Minus dimethyl-sulfoxide	110	10^{-2} M lactose	131
0.01 M dithio-threitol	92	10^{-4} M phenyl-β-D-thiogalactoside	90
pH 7	75	10^{-4} M *o*-nitro-phenyl-β-D-fucoside	106
pH 8	84	10^{-2} M glucose	112
		10^{-2} M galactose	36

A. Repressor (2 ng) was added to 200 ng of BrdU-λh80*dlac* [^{32}P]DNA in 0.3 ml of buffer I, containing 0.18 M KCl and adjusted to the various conditions indicated. UV irradiation (10 min) and IPTG treatment were done as in the legend of Fig. 2. Before filtration the reaction mixture was diluted with 3.0 ml of buffer I containing 50 μg of sonicated salmon-sperm DNA. Samples (1 ml) were filtered in triplicate.

B. The basic procedure for these experiments is described in the legend of Fig. 2. Buffer I containing 0.18 M KCl and the indicated ligand was used. For each ligand, an experiment with no repressor present during UV irradiation was done to establish that the ligand alone caused no filter retention of DNA.

prepared identically to the BrdU-λh80*dlac* DNA (the phages were from a double lysogen and separated in CsCl) and had the same degree of BrdU substitution (90%) as measured by buoyant density. Sucrose gradient centrifugation experiments established that the molecular weights of the BrdU-λh80 and BrdU-λh80*dlac* DNAs were the same. Therefore, these data provide strong evidence for specific attachment of *lac* repressor to *lac* operator. Another argument for specificity will be developed below when the action of effector ligands is considered.

Nonspecific Attachment. In low salt no evidence for specific cross-linking to operator is seen. Fig. 2*C* shows that in 0.01 M KCl, IPTG-stable complexes form equally well with BrdU-λh80 and BrdU-λh80*dlac* DNA. Without UV treatment, filter retention only of BrdU-λh80*dlac* DNA is observed. We interpret these results as follows: The *lac* repressor does have measurable general affinity for DNA and this affinity for nonoperator DNA is much higher in low salt (7). Although the affinity of repressor for nonoperator DNA is much less than for operator, the effective concentration of nonoperator-binding sites is very high. In low salt, the probability of repressor being bound to BrdU-λh80 DNA is very high (see ref. 7, and note that the affinity of repressor for BrdU-substituted DNA is about 10 times greater than for unsubstituted DNA). Apparently this weakly bound repressor is not able to cause filter retention of the DNA. After UV treatment and the formation of a more stable complex, the DNA is retained on filters.

Effector Ligands. Many effector ligands, mostly galactosides, are known to interact with the *lac* repressor and affect its affinity for operator. Some are inducers and decrease bind-

TABLE 2. *Stability of repressor-DNA complexes formed during UV irradiation*

Treatment after UV	Ratio of treated to control	
	Nonoperator (λh80 DNA)	Operator (λh80*dlac* DNA)
10^{-3} M IPTG[a]	1	1
1 M KCl[b]	0.95	0.95
80°, 30 min[c]	1.04	0.33
0.2 N NaOH[d]	0.82	—[d]
Pronase[e]	0	0

The procedure through irradiation was as described in the legend of Fig. 2. For the experiments with nonoperator [^{32}P]-DNA (BrdU-λh80), buffer I was used. For experiments where binding was to operator, BrdU-λh80*dlac* [^{32}P]DNA was used, and the buffer contained 0.18 M KCl. After UV irradiation (7.5 min), various treatments were given before filtration.

[a] At least 10 min before filtration, IPTG was added to a final concentration of 10^{-3} M. The control received no IPTG.

[b] KCl was added to a final concentration of 1 M. After at least 20 min at 25°, 3 ml of buffer I containing 50 μg of salmon-sperm DNA was added and 1-ml samples were filtered. The control was with water added in place of the KCl solution.

[c] λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, 400 μg of BSA was added (to protect from adsorption to the walls of the test tube) and the reaction mixture heated at 80° for 30 min. The control was not heated. λh80*dlac* DNA was not sonicated and only 0.5 ng of repressor was used.

[d] When nonoperator binding was studied, λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, NaOH was added to a final concentration of 0.2 N. The solution was incubated at 25° for 10 min and then neutralized with HCl. For the control, NaCl was added instead of NaOH. When operator binding was studied, the λh80*dlac* DNA could not be sonicated and such high backgrounds (no repressor) were obtained after denaturation as to render the experiment meaningless. Experiments where the DNA was renatured overnight at 65° were also unsuccessful because of high backgrounds.

[e] Pronase (20 μg) was added and the reaction mixture incubated at 37° for 10 min. The control got no Pronase, but was incubated at 37° for 10 min.

ing affinity; others increase affinity and are known as anti-inducers (12). IPTG, an inducer, is known to cause slight conformational changes in the *lac* repressor (15–17). Under conditions where specific binding to BrdU-λh80*dlac* DNA is seen, the presence of IPTG during irradiation completely eliminates the formation of stable complex (Fig. 2A and Table 1). It was conceivable that IPTG was acting, not by causing a conformational change in the repressor, but rather in some nonspecific way, perhaps by reacting with the free radicals produced during UV irradiation. Therefore, we checked the effect of other galactosides. As illustrated in Table 1 only ligands known to inhibit repressor binding to operator are effective. Anti-inducers such as glucose, *o*-nitrophenylfucoside, and phenylthiogalactoside (12) do not inhibit stable complex formation. Lactose, a disaccharide which recently has been shown to be an anti-inducer (18), does not inhibit, whereas melibiose, a disaccharide that acts as an inducer (12), greatly reduces the formation of stable complexes. These results establish that the effect of these ligands is mediated through the *lac* repressor.

In contrast, Fig. 3 shows that in low salt, where nonspecific interactions predominate, the presence of IPTG before and during irradiation does not affect the formation of stable complexes. This result is in keeping with our earlier observations that IPTG does not affect the binding of repressor to nonoperator DNA (7). Apparently, the conformational changes induced in the repressor by galactosides only affect binding to operator. Since in 0.18 M KCl, IPTG does eliminate stable complex formation, it follows that, under these conditions, attachment is to the *lac* operator.

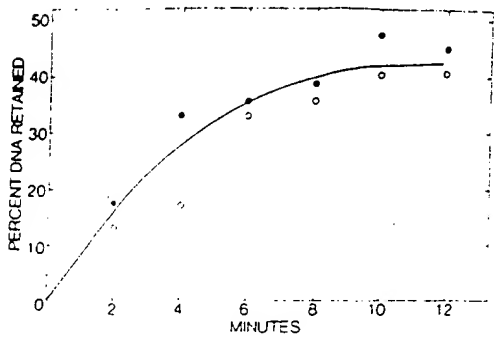

FIG. 3. The effect of IPTG on the photochemical attachment of *lac* repressor to wild-type BrdU-λh80 DNA. *Lac* repressor (1.0 ng) was added to 50 ng of BrdU-λh80 [^{32}P]DNA in 0.75 ml of buffer I. In one set of reaction mixtures, 1 mM IPTG was present. After 20 min at room temperature, the reaction mixtures were UV irradiated for the indicated times. To those samples with no ligand present during UV irradiation, IPTG was then added to a concentration of 1 mM. After an additional 10 min, 0.2-ml samples were filtered, in triplicate, through Millipore filters. (●——●) 1 mM IPTG present before and during UV treatment. (O- - -O) 1 mM IPTG added after UV treatment.

Chemistry and Efficiency of Attachment. Markovitz found that UV irradiation induces the formation of DNA polymerase–DNA complexes that are resistant to high salt, phenol, heat, and 0.1 M NaOH (3); he concluded that a covalent bond between DNA and protein had been established. Photoinduced protein–DNA complexes also are known to be resistant to SDS (2, 25). Under conditions where repressor is binding nonspecifically to BrdU-substituted DNA, we have obtained similar results (Table 2). The complexes also are stable to SDS (Table 3). These results strongly suggest that UV treatment covalently attaches repressor to DNA.

There is no reason to think that the mechanism of photochemical attachment of repressor to operator DNA is fundamentally different from attachment to nonoperator DNA. However, experiments to establish this point have proven more difficult, because under conditions where specific attachment of repressor to operator occurs, only one repressor protein is bound for each BrdU-λh80*dlac* DNA molecule (30×10^6 molecular weight). The filter assay requires that this protein cause the DNA to be retained on the nitrocellulose filters. Experiments where strand separation occurs (0.2 M NaOH and boiling temperatures) have not been successful. UV irradiation is known to introduce single strand breaks in BrdU-DNA, so after strand separation, the majority of DNA fragments would not be expected to have repressor peptides attached. Treatment at 80° causes a drop in DNA filter retention to a value about one-third that of the control. A denatured subunit attached to DNA may not be as effective in causing the DNA to be retained on the filters as the native tetramer. Although the above experiments were ambiguous, others were more definitive. The data in Table 3 provide

TABLE 3. *SDS and salt stability of photoinduced repressor–DNA complexes*

Reaction condition	DNA	Percent DNA in nonaqueous phase: No UV	+ UV
Nonspecific (0.01 M KCl)	λh80	0.8	62
Specific (0.18 M KCl)	λh80	0.3	1.4
	λh80*dlac*	0.5	8.2

For the experiment under nonspecific conditions, 200 ng of repressor was mixed with 400 ng of BrdU-λh80 [^{32}P]DNA in 1.5 ml of buffer I without dimethylsulfoxide. After 10 min of UV treatment, a modification of the procedure of Smets and Cornelis (25) was applied. BSA, SDS, and NaCl were added to a final concentration of 70 μg/ml, 0.2%, and 1 M, respectively; then an equal volume of $CHCl_3$:isoamyl alcohol (12:1) was added and the mixture gently shaken at 25° for 10 min. The water phase and $CHCl_3$ phase were separated and counted. The interphase was collected by filtration through Whatman GF/C glass filters. Before counting, the filters were washed with 1 N HCl containing 0.05 M sodium pyrophosphate, and then with ethanol. For the experiments under specific conditions, the same procedure was followed, except that only 8 ng of repressor was used and buffer I contained 0.18 M KCl.

strong evidence for SDS-stable cross-linking of repressor to operator. UV irradiation in the presence of repressor increases more than 10-fold the amount of DNA trapped in $CHCl_3$ and the protein–SDS interphase. Most important is the complete resistance of the photochemical complexes to high salt and inducer concentrations (agents that would quickly and completely eliminate normal repressor–operator complexes). Therefore, we think that photochemical attachment to operator occurs as it does to nonoperator DNA with the formation of extremely stable and probably covalent bonds.

Before UV irraidation, repressor causes a maximum of about 40% of BrdU-λh80*dlac* DNA to be retained on filters (Fig. 1). Fig. 2 shows that after UV irradiation and the formation of IPTG-resistant complexes, a maximum of about 20% of the DNA is retained. Therefore, from the data shown, it is apparent that the efficiency of complex formation is at least 50%. After 7.5 min. of UV treatment, we estimate that the sample has received a dose of 8×10^3 ergs/mm^2. Markovitz found that about 50×10^3 ergs/mm^2 was necessary to covalently attach DNA polymerase to poly(dA-dT) or normal *Escherichia coli* DNA (3). The difference in energy is in keeping with the increased sensitivity of BrdU-DNA to UV irradiation (2, 20).

As illustrated by the data shown in Table 1, the photochemical attachment of repressor to operator is much less at 0° than at 25°. This result has been obtained reproducibly and can only be partially compensated for by longer irradiation times. At 37°, background (no repressor) becomes higher and more variable. To obtain reproducible results, BSA must be present, presumably to protect repressor. However, the BSA must be heat treated (see below). Dimethylsulfoxide is not essential and can be eliminated. High concentrations of dithiothreitol do not interfere with photochemical attachment to operator.

Lac repressor binds strongly to poly(dA-dT) (7), and even more tightly to poly(dA-BrdU) (21). However, we found that

TABLE 4. *Photochemical attachment of the catabolite gene activator protein to BrdU-DNA*

Ligand	Exp. no.	Percent DNA retained: No UV, Treatment before filtering: None	No UV: KCl (1 M)	+ UV: None	+ UV: KCl (1 M)	+ UV: cGMP (3 mM)
None	1	10	0	64	60	—
	2	19	0	42	41	39
cAMP 0.3 mM	1	71	1	53	38	—
	2	45	1	44	26	29
cGMP 0.3 mM	1	—	—	—	—	—
	2	6	0	7	4	3

The reaction mixture contained the indicated ligand and 0.1 μg of BrdU-λh80*dlac* [^{32}P]DNA in 0.7 ml of buffer I. Enough CAP was added to cause 90% of the DNA to be retained on filters under our standard (no UV) condition (23). UV irradiation was for 10 min at 25° at a distance of 11 cm. After UV treatment, either KCl (1 M) or 3′:5′-cyclic GMP (3 mM) was added and the mixture was incubated at 25° for 20 min. Sonicated salmon-sperm DNA (50 μg) was added and then 2.5 ml of buffer I was added, and 1-ml samples were filtered in triplicate. In the case of no treatment before filtering, KCl or cGMP was omitted. Controls without CAP were done and subtracted as background. Neither cyclic AMP or cyclic GMP affected the background.

the photochemical attachment of repressor to poly(dA-BrdU) required higher UV doses than attachment to operator or to sonicated BrdU-λh80 DNA. A UV dose of about 50×10^3 ergs/mm^2 was required for 50% retention of [^{3}H]poly(dA-BrdU)–repressor complex on filters. Smith has observed that the rate of photochemical addition of [^{35}S]cysteine to poly(dA-dT) is considerably less than to calf-thymus DNA (19).

Photochemical attachment of repressor to normal λh80*dlac* DNA has not been successful. Initial experiments were done with intact λh80*dlac* DNA (30×10^6 daltons). Later we prepared DNA fragments of about 1×10^6 daltons, each of which contained the *lac* operator (22). With these operator enriched fragments, even 60×10^3 ergs/mm^2 gave no IPTG-resistant complexes.

Other Proteins. In this laboratory, we have studied the DNA binding properties of another regulatory protein, the catabolite gene activator protein (CAP, CR, or CGA protein) (23). The DNA-binding activity of CAP is stimulated by cAMP, but is eliminated by cGMP or by high salt (Table 4 and refs. 23 and 24). Binding specificity has not been demonstrated (23, 24). As shown in Table 4, UV treatment leads to the formation of a CAP–DNA complex that is stable to high salt and cGMP.

We also have confirmed that UV treatment will cause DNA polymerase and RNA polymerase to form salt resistant complexes with BrdU-DNA. All four DNA-binding proteins are effective in causing DNA filter retention at concentrations of 0.01 μg/ml or less (with or without covalent attachment). Other non-DNA-binding proteins (trypsin, ovalbumin, and BSA) will cause filter retention after UV treatment, but at least one 100-fold higher concentrations are needed (1 μg/ml or more). At 50 μg/ml, even BSA will cause filter retention unless it is heat treated (see *Methods*).

DISCUSSION

Markovitz (3) has shown that UV radiation covalently attaches DNA polymerase to poly(dA-dT) and to normal *E. coli* DNA. UV irradiation of BrdU-substituted DNA leads to debromination and the consequent production of highly reactive uracilyl radicals (2, 20). Therefore, it is reasonable to think that protein bound to DNA would be more readily attached to BrdU-DNA than to normal DNA. Smith (2) and Smets and Cornelis (25) have shown, in fact, that UV treatment of cells with BrdU-substituted DNA decreases the amount of DNA that can be extracted. They interpreted this as due to the formation of protein-DNA cross-links. After this paper was in preparation, we learned that Weintraub (26) has also recently obtained evidence for the cross-linking of proteins to BrdU-substituted DNA. He has found that a variety of proteins, including histones and RNA polymerase, can be attached to BrdU-substituted DNA from mammalian cells. If specific attachment occurs, involving the correct sites on the DNA and on the protein, then UV-induced cross-linking promises to be a very useful tool. However, in none of these studies was any evidence obtained for specific attachment.

We report here that the *lac* repressor can be photochemically attached to BrdU-substituted DNA and that in high salt (0.18 M KCl) the reaction is specific for *lac* operator, i.e., attachment occurs to BrdU-λh80*dlac* DNA but not to BrdU-λh80 DNA. Inducers of the *lac* operon (i.e., IPTG) prevent photochemical attachment of repressor to operator if they are present during UV irradiation, but not if added later. In low salt (0.01 M KCl), specificity is not observed; cross-linking occurs to BrdU-λh80 DNA as well as to BrdU-λh80-*dlac*, and IPTG has no effect. By analogy to previous work and from the stability of the repressor-DNA photoproduct we think that covalent bonds are formed.

We find BrdU-substitution to be essential; no attachment to normal λh80*dlac* is obtained even with much higher UV doses. There are large differences between normal and BrdU-substituted DNA in the types and numbers of photoproducts (20). These differences may be critical for the attachment of of sequence specific proteins. BrdU-substituted DNA may be advantageous, not only because of its greater photochemical reactivity, but also because the *lac* repressor binds tighter to BrdU-substituted DNA (8).

Cysteine adds photochemically to DNA (19) and Smith has shown that cysteine reacts with uracil to form 5-*S*-cysteine-6-hydrouracil (27) or with thymine to form 5-*S*-cysteine-6-hydrothymine (28). Eleven other amino acids photochemically add to uracil with cysteine, phenylalanine, tyrosine, histidine, lysine, and arginine being the most reactive (28). Polylysine also has been cross-linked by UV radiation to DNA (26). Since many amino acids can react, it seems likely that most DNA-binding proteins will be cross-linked by UV irradiation. We find that another DNA-binding regulatory protein, the catabolite gene activator protein (23, 24), also can be attached stably to DNA. We also have confirmed (26) that RNA polymerase can be cross-linked to BrdU-DNA.

Specific covalent attachment of DNA-binding proteins to their DNA substrate is obviously of great potential usefulness for identification of the DNA-binding sites of proteins and possibly in the isolation of the DNA region covered by the proteins. For mammalian cells especially, it may be useful to attach covalently chromosomal proteins to their specific sites by UV irradiation prior to disruption of the cell or nucleus. Perhaps less obvious is that covalent attachment may be useful for the *demonstration* of specific binding, which often is a major problem with DNA-binding proteins. For many proteins, binding specificity may be greatest in high salt. However, in high salt, DNA-protein complexes are detectable in sucrose gradients only if impracticably high concentrations of reactants are used; and the same is true for retention on nitrocellulose filters. By the methods described here, it is now possible to fix permanently the protein to DNA under conditions of maximum specificity.

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